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FACULTY OF PHARMACY

CENTER FOR PHARMACEUTICAL STUDIES

(cotutelle)

OCCURRENCE OF ANTIBIOTICS IN THE ENVIRONMENT

Doctoral thesis

2011 **Mgr. Marcela Seifrtová**

UNIVERZITA KARLOVA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

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FARMACEUTICKÁ FAKULTA

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Disertační práce

2011 **Mgr. Marcela Seifrtová**

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I declare that this thesis is my own work and all the sources of information used have been acknowledged.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

Marcela Seifrtová

ABSTRACT

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Department of Analytical Chemistry
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Title of Doctoral Thesis: Occurrence of antibiotics in the environment

The occurrence of pharmaceuticals in the environment is a growing concern. The pharmaceuticals are developed to have biological effects, and because of their biological and physico-chemical properties, their release into the environment can have impact in human health and ecosystems.

Despite their long half-life, pharmaceuticals are constantly being release into the environment, contributing to a continuous exposure of aquatic ecosystems.

The pharmaceuticals are considered as emerging environmental pollutants and the assessment of their environmental risk is acquired. Research on the occurrence, fate and effects is described in the scientific literature, however the data are still insufficient.

Antibiotics are an important class of pharmaceuticals widely used in human and veterinary medicine. The main problem associated with their presence in the environment is the development of mechanisms of resistance by bacteria, leading to the development of multi-resistant bacteria, which may put at risk public health in terms of effectiveness of the therapy.

The main focus of this doctoral thesis is on the development of new analytical methods for the determination of antibiotics from different classes in environmental waters.

The theoretical part of this doctoral thesis contains an introduction of the problematic of antibiotic residues in the environment, covering sources and pathways of antibiotics to the environment, fate, presence of antibiotics in environment and the legislation concerning the presence of antibiotics in environment. Another part deals with the analytical methods used for the determination of antibiotics in environment such as liquid chromatography with mass spectrometry. An important part the thesis is the overview of recently published analytical methods for the determination of antibiotics in scientific literature. This review was published in the internation scientific journal (chapter 4.1).

The practical part of the doctoral thesis can be divided into two areas. The first area deals with the determination of fluoroquinolone antibiotics in environment and with

the degradation of fluoroquinolone antibiotic pefloxacin by advanced oxidation processes. Newly developed methods for the analysis of fluoroquinolones were published in two international journals (chapter 4.2.1 and 4.2.2). The results of seasonal occurrence of antibiotics in wastewater and the efficiency of wastewater treatment plants are discussed in chapter 4.2.3. The degradation of fluoroquinolone antibiotic pefloxacin is described and discussed in chapter 4.2.4.

The second area of this doctoral thesis deals with the determination of tetracycline antibiotics in environment by different methods. The analysis of wastewater samples by high-performance liquid chromatography with fluorescence detection is described in one published work (chapter 4.3.1). The new methods developed by using ultra high-performance liquid chromatography are described in chapters 4.3.2 and 4.3.3.

The complete versions of published work are enclosed as supplements.

ABSTRAKT

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Název disertační práce: Výskyt antibiotik v životním prostředí

Výskyt léčiv a jejich metabolitů v životním prostředí je v posledních letech ve zvýšeném zájmu veřejnosti. Léčiva jsou látky určené mít speciální biologický účinek v organismu. Díky jejich fyzikálně-chemickým a biologickým vlastnostem mohou mít po uvolnění do životního prostředí důležitý vliv na lidské zdraví a na ekosystém.

Léčiva nejsou považována za perzistentní látky z hlediska jejich poločasu, ale jsou neustále uvolňována do životního prostředí díky používání v humánní a veterinární medicíně. To má za následek dlouhodobou expozici pro vodní ekosystém.

V současné době jsou léčiva považovány za velmi nebezpečné látky pro životní prostředí a je prováděn rozsáhlý výzkum týkající se jejich výskytu, osudu a efektů na životní prostředí a různé druhy organismů.

Antibiotika jsou velmi významná skupina léčiv široce využívaná v humánní a veterinární medicíně k léčení různých druhů infekcí. Hlavní problém výskytu antibiotik v životním prostředí je spojen s rozvojem antibiotické rezistence.

Předkládaná disertační práce je hlavně zaměřena na vývoj a validaci nových metod pro stanovení antibiotik v odpadních vodách.

Teoretická část této disertační práce se stručně zabývá úvodem do problematiky výskytu antibiotik v životním prostředí, zdrojích znečištění životního prostředí, osudem antibiotik v životním prostředí, problematikou antibiotické rezistence a legislativou týkající se antibiotik a léčiv. Další kapitola je věnována analytickým metodám využívaným k analýze vzorků životního prostředí, jako je kapalinová chromatografie s hmotnostní spektrometrií. Důležitá součást této disertační práce je vypracovaná rešeršní práce shrnující metody pro stanovení antibiotik v životním prostředí. Tato rešeršní práce byla publikována v mezinárodním odborném časopise (kapitola 4.1).

Praktická část této disertační práce je rozdělena do dvou tematických celků. První okruh se věnuje problematice stanovení fluorochinolonových antibiotik v odpadních vodách a dále studiu degradace fluorochinolonového antibiotika pefloxacinu pokročilými oxidačními procesy. Nově vyvinuté metody pro stanovení fluorochinolonů v odpadních vodách byly publikovány ve dvou zahraničních impaktovaných časopisech (kapitola 4.2.1

a 4.2.2). Výsledky analýzy vzorků odpadních vod z různých ročních období jsou diskutovány v kapitole 4.2.3. Kapitola 4.2.4 se věnuje degradaci fluorochinolonového antibiotika pefloxacinu.

Druhým okruhem diskutovaným ve výsledkové části je problematika tetracyklinových antibiotik a jejich stanovení pomocí kapalinové chromatografie s různými typy detekce. Nově vyvinutá metoda kapalinové chromatografie s fluorescenční detekcí je detailně popsána v publikované práci, která je opatřena krátkým komentářem (kapitola 4.3.1). Metody využívající kapalinou chromatografii za ultra vysokého tlaku s fluorescenční nebo hmotnostní detekcí jsou popsány v kapitolách 4.3.2 a 4.3.3.

Plné znění publikovaných prací jsou uvedeny v přílohách.

O RESUMO

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Título da Tese Doutoral: Ocorrência de antibióticos no meio ambiente

A ocorrência de fármacos no ambiente é uma preocupação crescente . Os fármacos são desenvolvidos de forma a possuírem efeitos biológicos específicos, e dadas as suas propriedades biológicas e físico-químicas, a sua libertação no ambiente pode ter impacto na saúde humana e nos ecossistemas.

Apesar do seu tempo de semi-vida, os fármacos estão constantemente a ser libertados no ambiente, contribuindo para uma exposição contínua dos ecossistemas aquáticos.

Os fármacos são considerados contaminantes ambientais emergentes, sendo por isso necessária a avaliação do seu risco ambiental. A investigação sobre a ocorrência, destino e efeitos encontra-se descrita na literatura científica, no entanto os dados são ainda insuficientes.

Os antibióticos são uma importante classe de fármacos, largamente utilizados em medicina humana e veterinária. O principal problema associado com a sua presença no ambiente é o desenvolvimento de mecanismos de resistência por parte das bactérias, levando ao desenvolvimento de bactérias multi-resistentes, o que pode comprometer a saúde pública em termos de eficácia da terapêutica.

O principal objectivo desta tese doutoral é o desenvolvimento de novos métodos analíticos para a determinação de diferentes grupos de antibióticos em águas ambientais.

A parte teórica contém uma introdução à problemática da presença dos resíduos de antibióticos no ambiente, abrangendo fontes e vias de contaminação, destino, ocorrência e legislação aplicável. Noutra secção encontram-se descritos os métodos analíticos usados na determinação de antibióticos no ambiente como, por exemplo, a cromatografia líquida acoplada à espectrometria de massa. Procedeu-se a uma revisão da metodologia analítica que se encontra descrita na literatura científica, e que sintetiza os métodos analíticos recentemente publicados para a determinação de antibióticos em amostras ambientais, e que foi publicada numa revista internacional (capítulo 4.1).

A parte prática desta tese doutoral pode ser dividida em duas secções. A primeira secção é dedicada à determinação de fluoroquinolonas no ambiente e à degradação da

pefloxacina por processos de oxidação avançada. Os novos métodos desenvolvidos para a determinação analítica de fluoroquinolonas foram publicados em dois jornais internacionais (capítulos 4.2.1 e 4.2.2). Os resultados da ocorrência sazonal de antibióticos em águas residuais e a eficiência das estações de tratamento são discutidos no capítulo 4.2.3. A degradação da pefloxacina foi também avaliada e encontra-se descrita e discutida no capítulo 4.2.4.

O segundo grande tema desta tese doutoral é a determinação de antibióticos do grupo das tetraciclinas em águas ambientais utilizando diferentes métodos analíticos. A análise de águas residuais por cromatografia líquida de alta eficiência com detecção por fluorescência é descrita num dos trabalhos publicados (capítulo 4.3.1). Os novos métodos desenvolvidos baseados na cromatografia líquida de alta eficiência são descritos nos capítulos 4.3.2 e 4.3.3.

As versões completas dos trabalhos publicados no âmbito desta tese doutoral encontram-se em anexo.

THE LIST OF ABBREVIATIONS

AOPs	advanced oxidation processes
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
APPI	atmospheric pressure photoionization
CE	capillary electrophoresis
CI	chemical ionization
CIPRO	ciprofloxacin
CTC	chlortetracycline
DC	direct-current
DEME	demeclocycline
DOXY	doxycycline
EI	electron ionization
EMEA	European Agency for the Evaluation of Medicinal Products
ENRO	enrofloxacin
ERA	environmental risk assessment
ESI	electrospray
eTET	epitetracycline
FD	fluorescence detection
FQs	fluoroquinolone antibiotics
FT-ICR	Fourier transform-ion cyclotron resonance
GC	gas chromatography
HETP	height equivalent to a theoretical plate
HPLC	high-performance liquid chromatography
ICH	Internal Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
LC	liquid chromatography
LIT	linear ion trap
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantitation
MALDI	matrix assisted laser desorption/ionization
MINO	minocycline
MIP	molecularly imprinted polymers
MLs	macrolide antibiotics

MS	mass spectrometry
MS/MS	tandem mass spectrometry
NOR	norfloxacin
O_3	ozonation
O_3/UV	combination of ozonation and UV photolysis
OFLO	ofloxacin
OXY	oxytetracycline
PEC	predicted environmental concentration
PEFLO	pefloxacin
PNEC	predicted no effect on environment concentration
QIT	quadrupole ion trap
QqQ	triple quadruple
RF	radio-frequency
SAs	sulfonamide antibiotics
SPE	solid-phase extraction
SPME	solid-phase microextraction
SRM	selective reaction monitoring
SST	system suitability test
TCs	tetracycline antibiotics
TET	tetracycline
TOC	total organic carbon
TOF	time-of-flight
UHPLC	ultra-high performance liquid chromatography
WWTP	wastewater treatment plant

CONTENTS

1. INTRODUCTION

Emerging contaminants are defined as compounds not currently covered by existing water-quality regulations that have not been studied before, and are thought to be potential threats to the environmental ecosystems and human health and safety. Nowadays, pharmaceuticals are classified as a group of emerging compounds. Antibiotics, followed by steroid compounds, analgesics/nonsteroidal and anti-inflammatory drugs, are the most widely studied classes of pharmaceuticals. Traditionally they have not been viewed as environmental pollutants. However given the intended use of pharmaceuticals, their fate characteristics and spectrum of effects, they might differ considerably from others micropollutants that have raised questions regarding their occurrence in the environment. Several studies have focused on their occurrence in the environment, and it is now recognized that pharmaceuticals are widespread pollutants in the aquatic environment. Limited knowledge is available about the implications of human exposure to a complex mixture of pharmaceuticals in environment.

Pharmaceuticals and their metabolites are continuously introduced into the aquatic environment at sub-therapeutic concentrations, posing a risk for aquatic organisms. Within this, antibiotics are one of the therapeutic classes that have received more attention from scientific community due to their presence in the environment being related to the development and spread of bacterial resistance, which may cause changes in ecosystems and compromise the effective treatment of diseases caused by pathologic microorganisms. Moreover, the use of antibiotics for veterinary purposes has been linked to the increased emergence of resistant strains of pathogenic bacteria that have potential to impact human health. Resistance genes and/or antibiotic resistant bacteria can be transferred from animals to humans. In addition, bacteria can develop cross-resistance between antibiotics used in veterinary medicine with those of similar structures used exclusively in human medicine.

Antibiotics used by humans enter sewage effluents *via* urine and faeces and by improper disposal. These antibiotics are discharged from private households and from hospitals. They are often partially metabolized after administration and a significant portion of antibiotics is excreted as the parent compound or in conjugated form which can be converted back to the parent antibiotic. Many researchers have shown the incomplete elimination of these compounds during the wastewater treatment process and residual amounts are released into the local aquatic surrounding. The existence of pharmaceutically active compounds, and in particular of antibiotics, in the aquatic environment, has been confirmed by several researches. The frequent occurrence of these compounds in surface and ground waters, a lot of which are used as source of drinking

water, may pose a problem to water utilities. Moreover, pharmaceuticals may persist in solid environmental matrices for a long time. The persistence depends on their photostability, binding and adsorption capacity, degradation rate and leaching into the water. Strongly sorbing pharmaceuticals such as tetracyclines and fluoroquinolones tend to accumulate in soils or sediment. In contrast to highly mobile pharmaceuticals such as sulfonamides have a potential to resist degradation and ted to leach into the groundwater and to be transported with the groundwater, drainage water and surface water run-off to surface waters.

The increased concern on the occurrence of antibiotic residues in the aquatic environment is related to the necessity of monitoring these compounds in the environment. The analysis of antibiotics in the environment represents a difficult task due to the high complexity of the matrices analyzed and to the very low concentrations (ng/L) at which target antibiotics are present in environmental waters. For this reason, sensitive and selective analytical methods are required for the monitoring and accurate quantitation of antibiotics in low ng/L concentration levels. The analysis of antibiotics in environmental samples has usually been carried out by high-performance liquid chromatography with mass spectrometry and to a lesser extent by ultraviolet or fluorescence detection. However, coupling liquid chromatography with mass spectrometry is a technique of choice for the analysis of environmental samples. Current trends in analytical chemistry are decreasing the analysis time, lowering solvent consumption and miniaturizing of equipment. The development in fast liquid chromatography has been primarily related to ultra-high performance liquid chromatography, monolithic columns, fused core columns, and high temperature liquid chromatography. Nowadays, the environmental analysis is employing those techniques for the determination of antibiotics and other compounds in environmental samples as well. Simultaneously, the development of an effective sample extraction and clean-up is mandatory as the environmental samples are very complex matrices and low concentrations of antibiotics are found. Off-line solid phase extraction is the most widely used technique for the sample preparation. It allows extraction, pre-concentration of analytes and clean-up of sample matrix in one step. However, some other techniques such as molecularly imprinted polymers and solid-phase microextraction has been used as well. Nowadays, a new trend became the injection of water samples directly onto high-performance of ultra-high performance liquid chromatography system with mass spectrometry detection excluding the usually time consuming sample preparation step.

This doctoral thesis deals with the occurrence of antibiotics in the environment and their determination in the environmental samples.

2. THE AIM OF THE WORK

The main aim of the experimental work presented in this doctoral thesis was to developed and evaluate new analytical methods for the identification and determination of antibiotics in environmental water samples.

The first task was to summarize recently published methods for the determination of antibiotics from different classes in environmental water samples.

The second part of this doctoral work was to develop new methods for the analysis of fluoroquinolone and tetracycline antibiotics in wastewaters. New methods based on liquid chromatography with fluorescence or mass spectrometry detection with solid-phase extraction as sample preparation, have been developed. The methods were validated and applied for the analysis of wastewater. Antibiotics were monitored during different season periods and seasonal influence and efficiency of treatment process in wastewater treatment plant was evaluated. The results (presented in five appended publications) highlight the potential utility of novel approaches for the determination of antibiotics in environmental waters.

The third task of this doctoral work was to study the degradation of fluoroquinolone antibiotic pefloxacin by advanced oxidation processes which may be included into advanced treatment processes in wastewater treatment plants in order to improve the treatment process.

3. THEORETICAL PART

3.1. Antibiotics in the environment

Antibiotics are an important group of pharmaceuticals in today's medicine. They are designed to have a specific mode of action and to stimulate a physiological response in humans, animals, bacterias or other organisms. Thus they could have toxic consequences for numerous exposed aquatic organisms. In addition to the treatment of human infections, they are also used in veterinary medicine.

Depending of the use of antibiotics, there are several ways of their entry into the environment. The main source of human antibiotics to the environment is by the effluents of wastewaters treatment plant (WWTPs). Antibiotics used in veterinary medicine are excreted via urine and faeces and enter the environment via manure that is applied to agricultural fields as fertilizer.

Of all emerging contaminants, antibiotics are probably the biggest worry because of their presence in the aquatic environment for a long time which leads to the appearance and the development of bacterial resistance.

Usually, antibiotics are present in the environment at low concentrations. Thus, sensitive and selective analytical methods are essential to allow their quantification in such complex matrices. At the same time, an effective sample extraction and clean-up is also required.

3.1.1. Sources of environmental contamination

Recent studies showed the occurrence of pharmaceuticals and antibiotics in wide range of environmental waters such as sewage effluents and surface waters, as well as in drinking water, surface and groundwater [1][2][3]. Moreover, antibiotics were detected in soil, sludge, manure and sediment [3][4][5].

The way how antibiotics enter the environment depends on the specific use of each compound. The physico-chemical properties of each compound determine its behavior in the environment. The entry of pharmaceuticals in general and antibiotics in particular, into the environment is due to their use in both human and veterinary medicine (Figure 1).

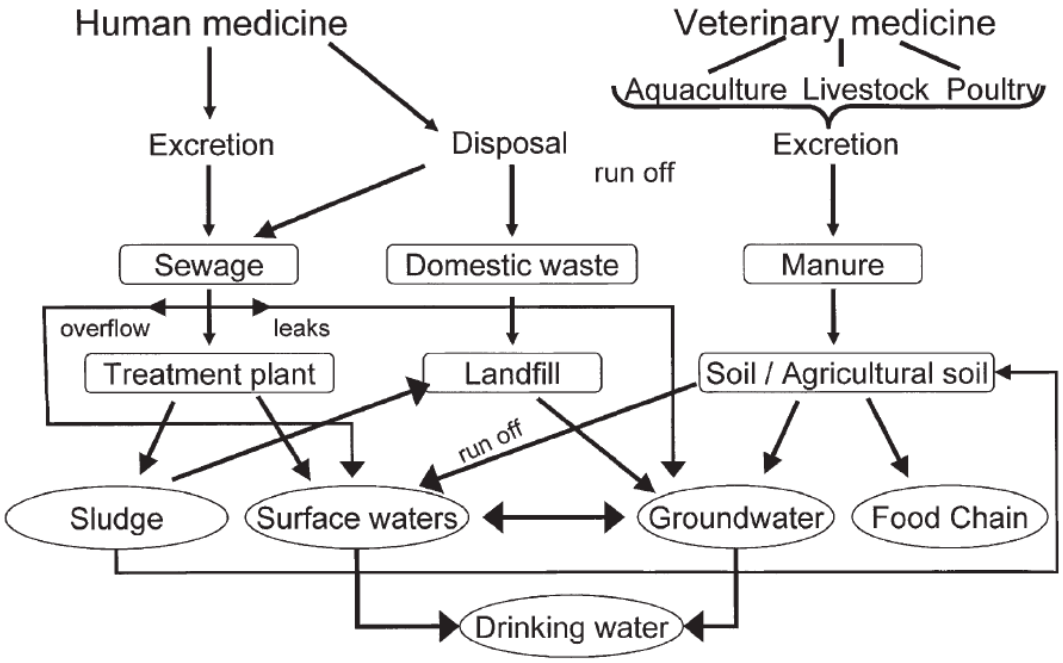


Figure 1: Sources of pharmaceuticals consumed in human and veterinary medicine into the environment [1].

In the case of antibiotics used in human medicine, a significant amount of antibiotics is excreted via urine and faeces in the form of the non-metabolized parent compounds or as their metabolites into wastewater. Presently, WWTPs are not designed to remove the most of the compounds completely and WWTPs are considered to be an important source of antibiotic contamination of surface and ground waters. During the treatment process antibiotics and their metabolites are only partially eliminated and residual amounts are released into the local aquatic surroundings depending on their properties, mobility and the persistence in the soil-water environment. More lipophilic compounds will be retained in sludge as a large part of elimination is achieved by adsorption on activated sludge which is partly mediated through hydrophobic interactions. In contrast, the polar antibiotics may not be eliminated effectively. Thus with the effluents antibiotic residues can get into the receiving surface waters including like creeks, rivers, or streams. In addition, antibiotics can reach surface waters by run-off from fields and agricultural areas where the sludge from WWTPs was dispersed as fertilizer. For this reason it is very important to monitor the effluents from WWTPs for the presence of antibiotics and other pharmaceuticals as well to improve the treatment processes.

Veterinary antibiotics are extensively used in livestock industry, poultry and fish breeding for the prevention and treatment of diseases (Figure 2). The antibiotics used for the treatment of animals in stables mostly end up in manure. The amounts of antibiotics excreted vary with the type of antibiotic, the dosage level, as well as the type and the age of animal [6]. The residues of antibiotics enter the environment directly when liquid

manure is sprayed on agricultural field. After an application of manure to farmlands, the residues of antibiotics can reach ground water via leaching or run-off from livestock slurries or can be accumulated in soil which delays their distribution. These excreted substances may also affect soil organisms.

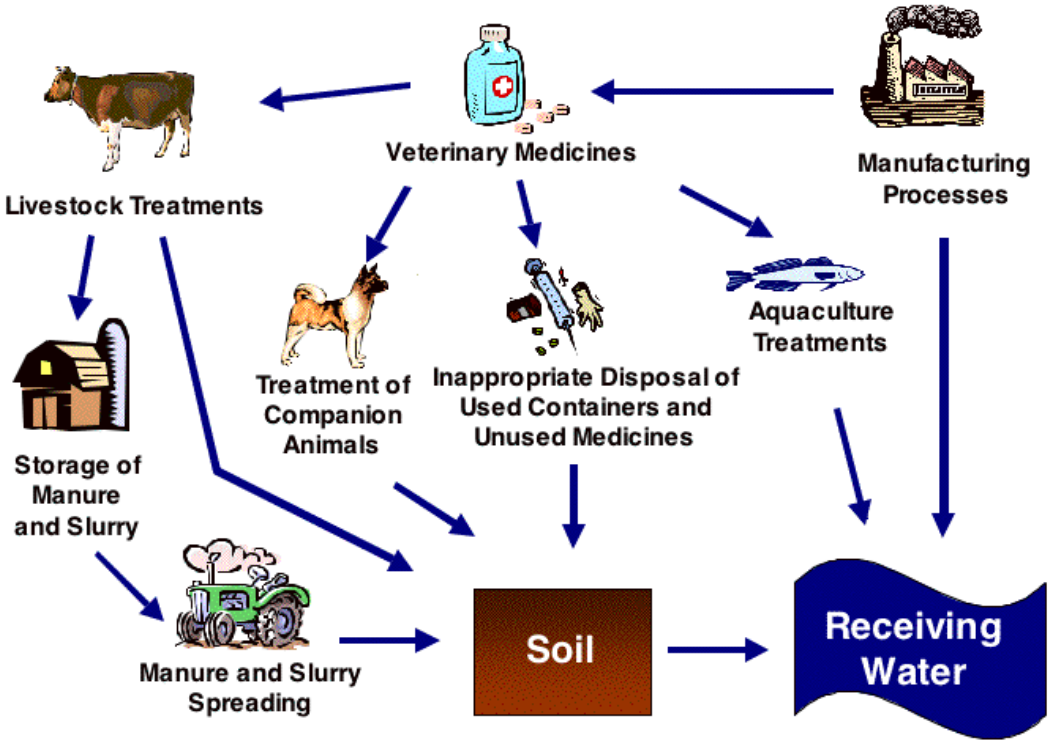


Figure 2: Routes of environmental exposure of antibiotics used in veterinary medicine [7].

In addition, antibiotics are extensively used in aquaculture. The main groups of antibiotics used are tetracyclines (TCs), sulfonamides (SAs) and fluoroquinolones (FQs) [8]. Antibiotics used in fish farms are exposed directly to the receiving waters as the most convenient method of treating fish with antibiotics is by the use of feed additives or by simple addition to the water. A parallel intake pathway results through excessive feed or excrements. Most of the unused drugs (overfeeding and loss of appetite by diseased fish) end up in the sediments where they are either degraded or slowly leach back into the surrounding water [8].

Another source of environmental contamination is disposal of unused medicine and expired drugs together with domestic waste (Figure 1). Hospitals, residential (private residences, dormitories, hotels, and residential care facilities) and commercial facilities are known contributors of antibiotics to municipal wastewater [9]. The manufactures and

industrial waste water have to be considered as another possible source of environmental contamination of surface waters.

However, hospital wastewaters are a significant source of contamination of by antibiotics [9][10][11][12]. The dilution of hospital effluents by municipal sewage will lower the concentration of antibiotics only moderately, because municipal waste water also contains antibiotic substances and disinfectants from households, veterinary sources and to a minor extent from livestock [13].

3.1.2. Occurrence of antibiotics in the environment

The occurrence of antibiotics in the aquatic environment is a risk for humans through drinking water and to aquatic organisms as well. Antibiotics have been detected in surface and ground waters in many countries around the world. However, studies of the fate and chemical behaviour of antibiotics in aquatic environments are rare [14]. Recent studies concerning of other pharmaceuticals residues in the aquatic environment have clearly shown that WWTPs are major contributors of pharmaceuticals in the environment. A number of antibiotics or their metabolites have been detected in sewage effluents, surface waters, ground water and in drinking water as well. Table 1 summarizes the recently published results of occurrence of antibiotics in environmental waters in EU. Macrolide antibiotic erythromycin together with sulphonamide antibiotic sulfamethoxazol and fluoroquinolone antibiotics belong among the most studied antibiotics in the environment.

The results obtained show the importance to continue in monitoring of antibiotics in environmental waters. Due to their high consumption, antibiotics along with their metabolites are continuously introduced to sewage waters, mainly through excreta, disposal of unused or expired drugs or directly from pharmaceuticals discharges [15]. Antibiotics were found in effluent from WWTP indicating the incomplete elimination during wastewater treatment process [16][17][18][19], and WWTPs are considered to be point sources of antibiotic contamination in surface and ground waters. The treatment efficiency of WWTP is affected by several factors, such as the physico-chemical properties of antibiotics, the treatment process, the age of the activated sewage sludge and environmental conditions such light intensity and temperature. However, generally the polar antibiotics may not be eliminated effectively, thus with the effluents, the remainders of the antibiotics can get into the receiving surface waters, like creeks, rivers, or streams. A large part of eliminations is achieved by absorption on activated sludge which is partly mediated through hydrophobic interactions. If the sludge from municipal WWTP dispersed on fields and agricultural areas contain adsorbed antibiotics it will affect the micro-organisms and can contaminate soil and ground water. For example, fluoroquinolones are eliminated in wastewater treatment by sorption to sewage sludge [20][21]. Their elimination rate is approximately 80-90% for ciprofloxacin and norfloxacin [20][22]. A partial elimination of macrolides in effluents from wastewater treatment plants has been reported [23][24] when clarithromycin, roxithromycin and erythromycin-H_2O have been found in treated effluents form three WWTPs. Their removal efficiency is above 50% [24] when activated sludge treatment in more efficient than chlorination and/or UV disinfection [24]. Ciprofloxacin, ofloxacin, sulfamethoxazol, trimethoprim and norfloxacin have been found in the secondary effluents from WWTPs [25]. The results of

comparison of different treatment processes and the concentrations of antibiotics found there shown lower concentration of antibiotics in the chlorination effluent than in the UV disinfection effluent.

As it has already been mentioned, hospital wastewater is a significant source of contamination of antibiotics [9][12][26][27]. The dilution of hospital effluents by municipal sewage will lower the concentration of antibiotics only moderately, because municipal wastewater also contains antibiotic substances and disinfectants from households, veterinary sources and to a minor extent from livestock [13]. In fact, some studies have demonstrated that bacterial resistance against FQs in the hospital wastewater is higher than that in the sewage treatment plant wastewater [26][28].

COUNTRY	ANTIBIOTIC	WWTP EFFLUENT (ng/L)	SURFACE/ RIVER WATER (ng/L)	REFERENCE
Spain	Erythromycin	-	up to 111.9	[29]
	Sulfamethoxazole	-	up to 119.3	
	Ofloxacin	-	up to 1903.6	
Italy	Erythromycin	up to 72	up to 8.12	[21]
	Clarithromycin	up to 145	up to 44.76	
	Sulfamethoxazole	up to 101	up to 2.39	
	Ofloxacin	up to 235	up to 18.06	
	Ciprofloxacin	up to 148	up to 37.50	
Sweden	Ofloxacin	up to 10	-	[30]
	Ciprofloxacin	up to 31.5	-	
France	Sulfamethoxazole	-	up to 26.0	[31]
	Ofloxacin	-	up to 10.5	
	Norfloxacin	-	up to 18.6	
Croatia	Erythromycin	up to 297	up to 162	[32]
	Clarithromycin	up to 1396	up to 26	
	Azithromycin	up to 1149	up to 8	
	Sulfamethoxazole	up to 4664	up to 9	
	Ciprofloxacin	up to 1304	-	
	Norfloxacin	up to 3131	-	
Portugal	Sulfamethoxazole	-	up to 53.3	[33][34][35]
	Ciprofloxacin	up to 309.2	up to 119.2	
	Norfloxacin	up to 35	-	
	Enrofloxacin	up to 211.5	up to 102.5	
Germany	Erythromycin	-	up to 22	[36]
	Clarithromycin	up to 520	up to 77	
	Roxithromycin	-	up to 16	
	Sulfamethoxazole	-	up to 93	
UK	Erythromycin	up to 830	up to 40	[37]
	Sulfamethoxazole	up to 12	up to 1	

Table 1: Examples of measured concentration of antibiotics in the aquatic environment in Europe.

The concern about antibiotic pollution leads to the extensive studies for the presence of antibiotics in the environment. For a short review of recently published results see Table 1. Antibiotics from different classes were analyzed. They include primarily sulphonamides (sulfamethoxazole), tetracyclines (tetracycline, chlortetracycline, oxytetracycline and doxycycline), macrolides (clatithromycin, erythromycin, roxythromycin) and quinolones and fluoroquinolones (ciprofloxacin, ofloxacin, norfloxacin).The concentrations of antibiotics measured in different countries are in the same range of concentrations in the different compartments. In general, higher concentrations (up to μg/L) are found in hospital effluents and the hospitals are considered as the major contributor of the environmental pollution. Municipal wastewaters and WWTPs are other important source of contamination as WWTPs are not able to eliminate all antibiotics completely. The concentrations of antibiotics in final effluent might be from μg/L to ng/L. In general, the lowest concentrations of antibiotics (only few ng/L) are found in different surface waters, ground waters and sea waters [21][31]. For example, some antibiotics such as ciprofloxacin, clarithromycin and erythromycin-H_2O were detected at concentration levels 5-20 ng/L in river waters in Italy [21]. Sulphametoxazole and norfloxacin were detected in rives at concentrations abou 20 ng/L [31]. The other antibiotics were at the pg/L concentration levels or undetectable.

The occurrence of antibiotics in the environment depends on the season and seasonal effects have been observed. For example, two times higher amounts of macrolide antibiotics were found during the winter season than in summer [23]. Such seasonal differences might have two reasons. Either, the elimination of macrolide antibiotics in WWTPs is smaller in winter due to lower biological activity, or the input of macrolide antibiotics is higher in winter. However, monthly sales data show that MLs are indeed sold in the times higher amounts in January/February than in summer because they are mainly used to cure infections of the respiratory tract. Seasonal influences on the frequency of detection of sulfamethoxazole and trimethoprim have been observed [38]. Especially, antibiotics were found in samples collected in fall, early winter, and late spring. These antibiotics are used for the first line of treatment of bacterial sinusitis, which is prevalent during fall, winter and spring seasons, and involves a combination of the above two antibiotics. Sulphonamide antibitiotics were detected infrequently in the early-summer samples. Trimethoprim was prevalent throughout the year and the same was true for tetracycline, which was detected in all but early-winter samples. Erythromycin-H_2O was detected more often in samples collected in the fall and early summer than in samples from other seasons. Different concentrations of antibiotics were found in China in both high (June) and low (March) water season [39]. The median concentrations of antibiotics in the high and low season ranged from 11 to 67 ng/L and from 66 to 460 ng/L, respectively. The reasons for the high water season might be because of storms water and

flooding, which may either input additional antibiotics form agricultural land and fish ponds to the river. A study from France confirmed the seasonal occurrence of antibiotics in river water samples. Clarithromycin was found only in winter samples while oxytetracycline has been found only in samples collected during spring [40]. Clarithromycin was determined at high levels (μg/L) in river water during the winter season in comparison to values reported for large European rivers. This might be explained by the small dilution of river water by background water [40].

Many authors developed methods for monitoring many antibiotics from different classes. These methods were applied to environmental samples. US Geological Survey on the occurrence of pharmaceuticals, hormones and other organic contaminants was performed during 1999 and 2000 in the US [41]. The concentration of 95 compounds was measured in water samples from a network of 139 streams across 30 states. Many compounds were detected in 80% of the streams sampled. The frequency of detection of at least one antibiotic was 22%. Another survey has been taken in the city Lausane and at the Lake Geneva in Switzerland [42]. A total of 58 pharmaceuticals, endocrine disruptors, pesticides and other emerging contaminants were assessed in WWTP in Lausane and in the effluent-receiving water body at Lake Geneva. All substances analyzed except one were detected in influent from WWTP or in the treated effluent. 40% of compounds analysed were also detected in raw drinking water.

A European survey on the occurrence of polar organic persistent compounds was recently performed [43]. A total of 164 ground water samples from 23 European Countries were collected and analysed for wide spectrum of analytes such as pharmaceuticals, antibiotics, pesticide, hormones, alkylphenolics, benzotriazoles, caffeine and other compounds. Of the total of 59 organic compound analyzed, nearly all were detected at least once. From the group of antibiotics, only sulfamethoxazol has been analyzed in ground water samples and found with a detection frequency of 24% at maximum concentration of 38 ng/L [43].

The positive detections of antibiotics, pharmaceuticals and other compounds in different type of environmental waters show the importance of monitoring of emerging contaminants in environmental samples to identify possible “hot spot” areas of pollution for protecting human health and ecosystem.

3.1.3. The fate of antibiotics in the environment

After entering of antibiotics into the environment their fate depends mainly on the structure and physico-chemical properties of the compound (such as molecular structure, size, shape, solubility, speciation and hydrophobicity), climatic conditions, type of matrix and variety of other environmental factors. Once the emerging contaminants are released into the environment, they can be subjected to different processes that contribute to their elimination and decrease of their concentration. These processes such as sorption, biotic and abiotic transformation, chemical and photochemical degradation, biodegradation, but also natural attenuation directly influence the fate and transport of antibiotics in the environment as well as their biological activities. Depending on the type of compartment in which antibiotics are present (such as groundwater, surface water, sediment, soil or WWTPs) they can undergo different transformation processes forming products of different environmental behavior and ecotoxicology profile. However some compounds are inert and can persist in the environment and are difficult to degrade. These compounds can be toxic and accumulate in food chain which is a risk for public health [44].

Drugs used by humans are discharged to sewer systems via urine and faeces, and then go to WWTPs. There the substance can undergo different processes [45]:

- The substance can be ultimately mineralized to carbon dioxide and water.
- The drug can be retained in the sludge in dependence of its lipophilicity, other binding properties and persistence, a part of the drug.
- The substance can undergo metabolization to a more hydrophilic form and it will pass the WWTP and can easily reach the aquatic environment.

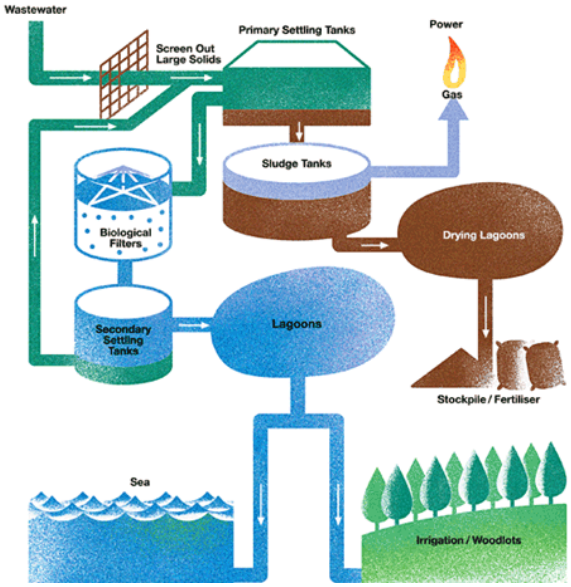


Figure 3: Scheme of wastewater treatment plant [46].

In WWTP, elimination rates differ considerably depending on the compound, environmental conditions, and process conditions. Generally, two elimination processes taking place during wastewater treatment are important for the removal of contaminants: adsorption to suspended solids (sewage sludge), and biodegradation (Figure 3). Adsorption is dependent on both hydrophobic and electrostatic interactions of the compound with particulates and microorganisms. In case that pharmaceutical occurs mainly in the dissolved phase, biodegradation is probably the most important elimination process in WWTP. It can occur in aerobic zone in activated sludge treatment, or anaerobically in sewage sludge digestion. In general, biological decomposition of micro-pollutant including pharmaceuticals increases with the increase of hydraulic retention time and with age of the sludge in the activated sludge treatment [47]. However, hydraulic retention times are generally shorter than the degradation half-lives of most antibiotics entering WWTPs. As a result, polar compounds are discharged into the effluents before complete degradation occurs. Moreover, the polar antibiotics may not be eliminated effectively in WWTPs, as a large part of elimination is achieved by adsorption on activated sludge, which is partly mediated through hydrophobic interactions. For this reason, antibiotic residues are expected to be found in surface waters. By contrast, rather lipophilic compounds are more easily removed from the wastewater by adsorption on the sewage sludge through hydrophobic interactions. TCs and FQs are effectively removed by retention in sludge through precipitation as metal complexes and by adsorption through hydrophobic interactions, respectively [1]. For this reason, there is risk for microorganisms and further contamination of soils and groundwater when the sludge from WWTPs containing antibiotics is dispersed on fields and agricultural areas.

Moreover, microorganisms can play an important role in elimination of emerging pollutants during water treatment. The contaminants can be microbially transformed in WWTPs and less potentially harmful compounds are then released into the aquatic environment. The biological tank of WWTPs largely comprises bacteria but it also has an important protozoan flora, so that synthetic chemicals can be degraded [44]. However, antibiotic might have negative effect to bacteria in WWTP as well.

Once, the antibiotics reach the aquatic environment, a hydrolysis is an important degradation pathway. Another abiotic transformation is photodegradation that is significant for the organic pollutants in the surface layers of water bodies that receives appreciable amount of sunlight. These photolytic reactions are often complex and lead to multiple degradation products which are also of concern, because they may have toxicity similar to or even higher than the parent compounds [44]. TCs and FQs belong to antibiotics the most susceptible to photodegradation [48]. TCs can persist for relatively long periods if the sunlight is not present.

Concerning the persistence of antibiotics in the environment, strongly sorbing antibiotics tend to accumulate in soil or sediment throughout a wide range of environmental conditions. TCs indicate strong sorption to clay materials, soils and sediments and/or sewage sludge. They readily precipitate with cations such as calcium [9]. FQs and macrolides (MLs) show a significant sorption [48]. By contrast, highly mobile pharmaceuticals (e.g. SAs and aminoglycosides) exhibit weak sorption to soil and activated sludge [48] and tend to leach into groundwater and be transported with groundwater, drainage water, and surface run-off to surface waters [49].

3.1.4. Effects of antibiotics and antibiotic resistance

Of all the emerging contaminants, antibiotics are the biggest concern since their environmental occurrence can increase the proliferance of resistant bacteria (Figure 4). This causes a serious threat for public health as more and more infections can no longer be treated with the presently known antibiotics. Antibiotics are expected to occur in trace concentrations, although they are continuously released into the environment [50]. Moreover, the long-term presence of low levels of residues in foodstuffs has led to the problem of drug resistance in pathogens in human body [51]. Although the antimicrobials given to humans are often not the same as those given to animals, the structures can be similar enough. Therefore the antimicrobials used for animals can induce resistance to those used for humans. Overall, more than 70% of the bacteria are insensitive against at least one antibiotic, and many show multiple resistance patterns [52].

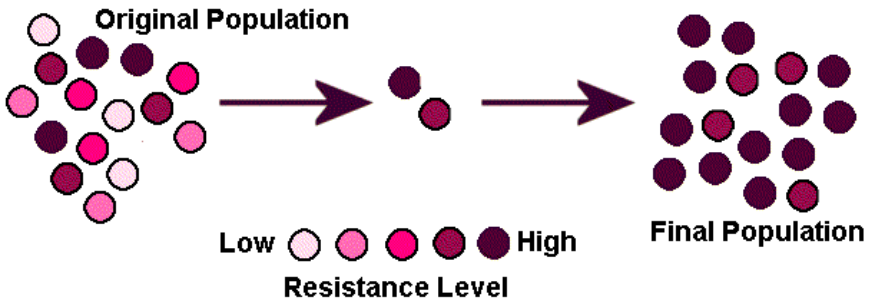


Figure 4: A schematic of the development of antibiotic resistance [53].

There is a serious lack of information regarding the development of antibiotic-resistance in bacteria due to the frequent use and exposure to lower concentrations of antibiotics since they accumulate in the environment. Antibiotics may have qualitative and quantitative effects upon the resident microbial community of sediments. Concentrations below therapeutic levels may play a role in the selection of resistance and its genetic transfer in certain bacteria [13]. The persistence of the antibiotics residues may assist in maintenance or development of antibiotic resistant microbial population. Antibiotic resistant strains reach the environment through manure and liquid manure of animals as well as through human excretions. The cyclic application e.g. of manure on the same location may result in the continuous exposure of soil microbes to antibiotic residues and increase the emergent antibiotic resistant bacteria population [54]. Resistant bacteria are also found in the faeces of healthy persons and the intestinal flora of healthy individuals increasingly serves as a reservoir of bacteria with multiple resistance [55]. For example, isolated *E. coli* strains were tested for their resistance patterns against 24 different antibiotic compounds. Investigations have been done in sewage, sludge and receiving

water from different WWTPs. The study showed the highest resistance rates in *E. coli* strains of a WWTP which treats not only municipal sewage but also sewage from hospital. Resistant *E. coli* strains were not only found in the wastewater and sewage sludge, but also in the receiving water. The highest resistance rates were found for tetracycline (57%) which is antibiotic used in medicine for decades. Resistance rates have been observed both in Gram-negative bacteria as well as in Gram-positive cocci. From the group of quinolone antibiotics, resistance rate for nalidixic acid was up to 15%, for sulfamethoxazol/trimethoprim up to 13%. More recent FQs norfloxacin, ciprofloxacin and ofloxacin were shown to be almost 100% effective against bacteria [55].

Concerns about bacterial resistance and particularly the emergence of multiple antibiotic resistance, especially associated with antibiotics that were used both in human and veterinary medicine, led in the United Kingdom to the setting up of a Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine [56]. This committee recognized that the administration of antibiotics, particularly in sub-therapeutic doses, posed certain hazards to human and animal health. The committee concluded that these hazards could largely be avoided and recommended that antibiotics available without prescription in animal feed should be of economic value in livestock production, but should have little or no application as therapeutic agents in man or animals and should not impair the efficacy of prescribed therapeutic drugs through the development of resistant strains of the organism. Therefore penicillin and TCs were no longer permitted at sub-therapeutic levels for growth promotion, and they became available only on veterinary prescription at therapeutic levels [56].

3.1.5. Usage and legislation

Pharmaceuticals were in generally ignored as environmental contaminants until the early 1990s. Since then the environmental risk caused by pharmaceuticals became an important question in environmental sciences mainly due to high number of studies which detected pharmaceuticals in the environment. Numerous studies on fate and behavior of pharmaceuticals in the environment clearly demonstrated the possible environmental impact. However, there is a gap in legislation regarding the environmental contamination by pharmaceuticals. The reason might be still little knowledge about their biological effects on aquatic environment as well as missing conclusive studies concerning chronic toxicity [57].

Antibiotics may be divided into antibiotics used in human or veterinary medicine. They are the primary therapeutic group of pharmaceuticals studied in the world. In Europe, two thirds of all antibiotics are used in human therapy and one-third for veterinary purposes. In human medicine, antibiotic pose the third biggest group among all pharmaceuticals making up more than 60% of all prescriptions [1]. In human medicine ß-lactams, SAs and FQs are the most often prescribed groups of antibiotics (Figure 5). TCs, aminoglycosides, and other antibiotics are used in smaller quantities compared to the above four antibiotic groups [48].

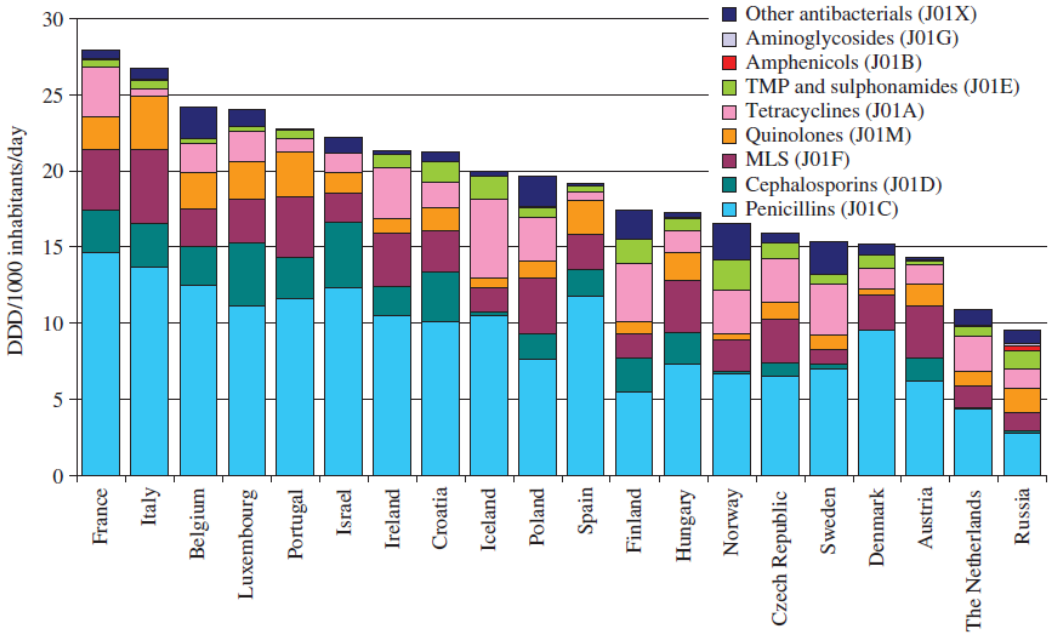


Figure 5: Outpatient antibiotic use in 20 European countries in 2006 [58].

In veterinary medicine, more than 70% of all consumed pharmaceuticals are antibiotic agents [1]. The veterinary antibiotics may be subdivided into substances used as prophylactic agents and therapeutics for treatment. The antibiotic compound classes

primarily administered in veterinary medicine are TCs, followed by SAs, ß-lactams, aminoglycosides, and MLs, FQs and others [54]. Specifically, cattle, poultry and pigs are treated with the majority of antibiotics, while all other domestic animals received only 1% of prescription within the European Union (EU). However, sale and/or use data of veterinary antibiotics are currently lacking in the public domain. Available data on use and sale trends of veterinary antibiotics in the world is poor and incomplete as there has never been systematic collection of data based on a standard procedure. Only recently a few EU member states (the Scandinavian countries and Netherlands) have started to collect data on the use of antibiotics [54].

In 1992 the Commission Directive 92/18/EEC lay down the requirement for an environmental risk assessment (ERA) which should be included in an application for marketing authorization for veterinary pharmaceuticals [59]. For this purpose, the European Agency for the Evaluation of Medicinal Products (EMEA)/Committee for Veterinary Medicinal Products (CVMP) published a document to provide a guidance on the evaluation of the potential risks of exposure of veterinary medicinal product, its ingredients and relevant metabolites to the environment and the assessment of potential harmful effects which the use of the product may cause to the environment (EMEA/CVMP/055/96-FINAL) [60].

Later the European Commission extended its concern to pharmaceuticals for human use. According to Article 8 of Directive 2001/83/EC as amended by Directive 2004/27/EC an application for a marketing authorization of a medicinal product for human use shall be accompanied by an evaluation of potential environmental risks caused by the medicinal product [61]. Any risk relating to the quality, safety or efficacy of the medicinal product regarding human health may influence its marketing authorization. However, undesirable effects on the environment are not taken into consideration for a final decision and do not prevent any medicinal product application of being authorized. Thus, in current procedures, a negative environmental risk assessment can not affect the risk-benefit balance of a medicinal product [57]. As a consequence the EMEA/Committee for Medicinal Products for Human Use (CHMP) has released a guideline on ERA of medicinal products for human use (EMEA/CHMP/SWP/4447/00) [62]. An ERA is required for all new marketing authorization application for a medicinal product and also if there is an increase of the environmental exposure. ERA of both veterinary and human pharmaceuticals is normally conducted in two phases. The first phase (Phase I) estimates the exposure of the environment to the medicinal product or its metabolites. The estimation is based only on the drug substance, irrespective of its route of administration, pharmaceutical form, metabolism and excretion. The Predicted Environmental Concentration (PEC) is calculated and this parameter corresponds to the estimation of exposure concentration in wastewater. If the PEC value is equal or above 0.01 µg/L further

assessment described in Phase II should be performed. In some cases, the PEC value cannot be applicable when the substances (e.g. synthetic estrogens) demonstrate effect on the reproduction of vertebrate or lower animals even at lower concentration than 0.01 μg/L and these substances should enter the Phase II evaluation. In the second phase (Phase II), information about the fate and effects in the environment is obtained. Phase II is divided in two tiers (A and B). Tier A involves tests of toxicity for algae, Daphnia and fish, fate tests such as evaluation of the PEC/PNEC (Predicted no Effect on the Environment) ratio, and water sediment study. It the ratio PEC/PNEC is higher than 1 for surface water/groundwater and/or above 0.1 for PNEC microorganisms potential environmental impact is indicated. Further evaluation is needed and the assessment continues in Tier B. Tier B focuses on the evaluation of effects on specific microorganism and terrestrial additional species within environmental compartments that are likely to be affected.

In the case if there is any potential environmental risk presented by the medicinal product, all information, precautionary and safety measures has to be described in the Summary Product Characteristics and Package Leaflet for professional and patient information and no refusal is accomplished. Moreover, the recommended labeling should include general statement:

> *"Medicines should not be disposed of via wastewater or household waste. Ask your pharmacist how to dispose of medicines no longer required. These measures will help to protect the environment."*

Concerning the residues of veterinary pharmaceuticals, the EMEA guideline sets a threshold value of 0.1 mg/kg for residues of veterinary pharmaceuticals in soils. However, the threshold is applicable only for the approval of new substances. In 1998 the European Commission banned the use of medically important growth promoters like bacitracin, spiramycin, tylosin, and virginiamycin (related to Synercid). With regard to the use of antibiotics as growth-promoting feed additives, the Commission proposed a phasing out by 2006 [6] and finally the use of antibiotics as growth promoters was banned in the EU from 1st January 2006 [54].

3.1.6. Fluoroquinolones

Quinolones are the most powerful and important classes of synthetic antibiotics in human and veterinary medicine. Fluoroquinolones (FQs) are piperazinyl derivates of the quinolone nadixilic acid and represent the second generation of this family of antibiotics [63]. FQs are characterized by fluorine atom at position 6 of the quinolone naphthyridine or benzoxazine ring (Figure 6). They have two relevant ionisable functional groups, the 3-carboxyl group and N-4 of the piperazine substituent. Therefore FQs have two pK_a values and their acid–base behavior will be significantly affected by physicochemical properties of the solvent. Reported values of pK_a for carboxylic group range from 5.7 - 6.3, whereas those for protonated amino group are higher (7.6 - 8.3). The intermediate form of FQs is a zwitterion [63].

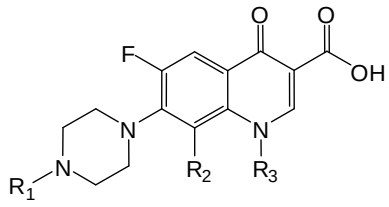
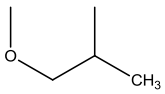
Compound	R1	R2	R3	General structure
NOR	-H	-H	$-C_2H_5$	
CIPRO	-H	-H		
PEFLO	$-CH_3$	-H	$-C_2H_5$	
OFLO	$-CH_3$	-H		
ENRO	$-C_2H_5$	-H		

Figure 6 : Chemical structures of selected FQs: norfloxacin (NOR), ciprofloxacin (CIPRO), pefloxacin (PEFLO), ofloxacin (OFLO) and enrofloxacin (ENRO).

FQs are effective against Gram-negative but also moderately active against Gram-positive bacteria [63]. Their activity is based on the inhibition of the enzyme DNA gyrase or topoisomerase II, which are responsible for the replication, transcription and reparation of the bacterial DNA and the separation of daughter DNA strands during bacterial cell division [64] Nowadays, they are broadly used in the treatment of a wide variety of infection diseases (respiratory diseases and enteric bacterial infections, and they are also alternative agents for the treatment of many sexually transmitted diseases, as well as osteomyelitis [10][64], because of their broad spectrum activity and good per oral absorption [28].

FQs are generally prescribed to the patient at the dose between 300-600 mg per day for the therapeutic treatment. Administered FQs are almost excreted as unchanged compounds in urine, for example 45-62% of CIPRO and 20-40% of NOR was excreted in the urine within 24 hours of administration and significant amounts were also excreted in faeces, for example 15-25% of CIPRO and 28% of NOR [65].

FQs are rather resistant to microbial degradation and may persist in the environmental waters because of their strong sorption properties. They can adsorb onto sewage sludge, soils and sediments to a significant extent [13]. For FQs, sorption to sewage sludge has been suggested as the primary removal mechanism during secondary wastewater treatment [29]. On the other hand, degradation of these antibiotics, including photolysis [66] and chemical oxidation [67] may be significant for the environmental ecosystem if they are not adsorbed to sediments in the aquatic environment. Effluence of FQs into the environmental waters occurs mainly as the parent compounds and secondly as a consequence of inadequate treatment of human and animal excreta [28].

3.1.7. Tetracyclines

Tetracyclines (TCs), produced by *Streptomyces* spp. are broad-spectrum bacteriostatic agents highly effective against numerous Gram-positive and Gram-negative bacteria. Their basic structure consists of a hydronaphthacene backbone containing four fused rings (Figure 7). Various analogues differ primarily by substitutions of the fifth, sixth or seventh position on the backbone [18]. Three pK_a values associated with TCs have several implications for method development. Throughout the range of pH, TCs always possess a local charge, and are zwitterionic in the approximate range of pH 3 to 9. Under alkaline conditions, they assume a confirmation that allows for hydrogen bonding between N_4 and OH_{12a}. Under neutral and acidic conditions, the N_4 position becomes protonated, disrupting the previous conformation, and a hydrogen bond interaction occurs with O_3. TCs are also able of assuming several other conformations depending on their environment.

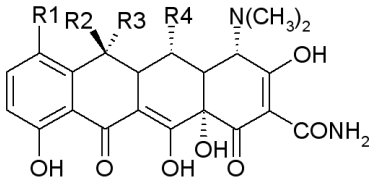
Compound	R_1	R_2	R_3	R_4	General structure
CTC	-Cl	-OH	$-CH_3$	-H	
DOXY	-H	-H	$-CH_3$	-OH	
OXY	-H	-OH	$-CH_3$	-OH	
TET	-H	-OH	$-CH_3$	-H	
DEME	-Cl	-OH	-H	-H	
MINO	$-N(CH_3)_2$	-H	-H	-H	

Figure 7: Chemical structures of selected TCs: chlortetracycline (CTC), doxycycline (DOXY), oxytetracycline (OXY), tetracycline (TET), demeclocycline (DEME) and minocycline (MINO).

TCs act by binding to bacterial ribosomes to prevent tRNA access the receptor sites and this leads to protein synthesis inhibition. TCs are used in human and animal medicine. In human medicine TCs are useful in the treatment a broad range of infections like *Mycoplasma* or *Chlamydia* [18]. They are widely used in livestock because of their therapeutic and economic advantages. They are used both in the treatment and in the prevention of animal infections [50]. TCs are rapidly absorbed from the gastrointestinal tract and widely distributed in the body. They undergo minimal or no metabolism and they are excreted in urine and faeces unchanged or in a microbiologically inactive form.

TCs are unstable in basic media, relatively stable in acidic media, and form salts in both media. They have been found to form complexes with chelating agents, such as divalent metal ions and strongly bind to proteins and silanol groups. This complexation influence effective extraction and further separation. Moreover, the complexation has an effect on the spectral characteristics of TCs. The complexes of TCs with metal ions show dramatically increase the fluorescence intensity of the molecules, particularly when

complexed with Mg^{2+} a Ca^{2+}. Chelation with multivalent cations can occur at A ring or BCD ring systems. Multiple species of chelated TCs can co-exist in solution. The number and kind of species can change depending on pH or on type of metal. Different TCs can behave in a different way under the same conditions [18][69]. Their presence in soils and in sewage sludge is a result of their complexation with cations. For this reason they are very often found in sewage sludge and in soils. They were detected not only in wastewater, but also in surface water. TCs are photodegradable. Therefore it is an important step of removal process when the sunlight is present [70]. TCs can undergo epimerization in solution to 4-epitetracycline (eTET), which has much lower antibiotic activity. However, under specific alkaline conditions and in the presence of a complexing metal, the eTET can convert back to TET.

3.1.8. Advanced oxidation processes

As it was mentioned before, WWTPs are not able to remove all pharmaceuticals completely. Advanced oxidation processes (AOPs) are promising tools for the removal of persistent pharmaceutical compounds from water. Photolysis and ozonation are promising alternatives among AOPs and the efficiency of treatment process can be enhanced by their combination. AOPs are able to generate hydroxyl radical which is the strongest known oxidant and it is able to oxidize and mineralize almost every organic molecule into CO_2 and inorganic ions [71]. Ozonation can also be seen as an AOP because the unique feature of ozone is its decomposition into reactive hydroxyl radicals. It can be used in advanced treatment of secondary wastewater effluent as it has potential to disinfect primary wastewater effluents [72]. Ozone is unstable in water and its stability largely depends on the water matrix, especially its pH, the type and the content of natural organic matter and its alkalinity.

The pH of water is important because hydroxide ions initiate ozone decomposition which involves the following reactions [73]:

$$O_3 + OH^- \rightarrow HO_2^- + O_2 \qquad \text{eq. 1}$$

$$O_3 + HO_2^- \rightarrow {}^{\bullet}OH + O_2^{\bullet -} + O_2 \qquad \text{eq. 2}$$

$$O_3 + O_2^{\bullet -} \rightarrow O_3^{\bullet -} + O_2 \qquad \text{eq. 3}$$

According to reactions eq. 1 and eq. 2 the initiation of ozone decomposition can be artificially accelerated by an increase of pH or by an addition of H_2O_2 [73]. It is expected that molecular ozone is the major oxidant at acidic pH (eq. 4 and 5), whereas less selective and faster radical oxidation (mainly hydroxyl radical) becomes dominant at pH > 7 as a consequence of OH^- accelerated ozone decomposition (eq. 6 and 7).

$$pH <\approx 8: O_3^{\bullet -} + H^+ \leftrightarrow HO_3^{\bullet} \qquad \text{eq. 4}$$

$$HO_3^{\bullet} \rightarrow {}^{\bullet}OH + O_2 \qquad \text{eq. 5}$$

$$pH >\approx 8: O_3^{\bullet -} \leftrightarrow O^{\bullet -} + O_2 \qquad \text{eq. 6}$$

$$O^{\bullet -} \rightarrow {}^{\bullet}OH + OH^- \qquad \text{eq. 7}$$

Since the oxidation potential of hydroxyl radicals is much higher than that of ozone molecule, direct oxidation is slower than radical oxidation and furthermore causes incomplete oxidation of organic compounds [74]. Organic molecules are oxidized with ozone selectively. Ozone reacts mainly with activated aromatic system, double bonds, non-protonated amines and sulfidic groups [73].

3.2. Determination of antibiotics in the environment

The widespread occurrence of antibiotics in the environment has led to an increase of investigation of the fate and effects of antibiotics on aquatic organisms and humans as well. For these studies it is important to carry out monitoring of antibiotics in the environment. As a consequence new analytical methods have been developed. The sensitivity and possibility of selective identification and quantification of antibiotics in the environment are the basic requirements of such methods. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) is a method of choice to assay antibiotics together with their metabolites in environmental analysis. Solid-phase extraction (SPE) is used for sample preparation as it is easy and suitable for the preparation of environmental samples.

3.2.1. Sample preparation

Environmental samples are very complex matrices and they are not ready for direct introduction into analytical instruments. Thus before the proper analysis a sample preparation step is necessary. This step is crucial in environmental analysis as it is necessary to pre-concentrate analytes in sample, to remove interferences from matrix and to prepare analytes in suitable form for subsequent chromatographic analysis ideally in one step. Usually, the sample preparation is one of the most time-consuming step during the whole analysis and it can be a source of imprecision and inaccuracy for all the analysis. Concerning the sample volume used, traditionally 500 or 1000 ml of surface or ground water was used. When the wastewater is analyzed, lower sample volume might be used as the antibiotics occur there in higher concentration levels. Nowadays, the trend is using smaller sample amount in order to reduce time necessary for sample preparation step. However, a problem with matrix interferences may arise as wastewater samples are complex matrices. When surface or ground waters are analyzed, using smaller sample volume require a sensitive detection as the analytes occur there in very low concentration levels (ng/L) [75].

In environmental analysis the sample preparation procedure usually includes filtration of sample, pH adjustment of sample, addition of chelator and final preparation. The filtration of sample before the proper sample preparation and extraction step is suggested to remove particles from the water samples which may plug up the SPE cartridges and thus slow down the sample preparation step. However, not much attention has been paid to pharmaceutical residues which are hydrophobic and bound to suspended particles in water samples. In this case, filtration might lead to loss of the analytes for subsequent analysis. The pH of sample solution is very important as it influences the

chemical form of analytes in sample, their stability and their interaction between the analyte and SPE sorbent material. Addition of chelator such as EDTA is important during the analysis of TCs and FQs as they are able to form complexes with divalent and polyvalent cations. The formation of these complexes decreases the extraction efficiency [75][76].

SPE is the mostly used sample preparation procedure in environmental analysis allowing the enrichment of analytes and clean-up of the sample in one step. SPE has several advantages such as high recovery, effective pre-concentration of analytes, need of less organic solvents in comparison with liquid-liquid extraction (LLE), easy operation and possibility of automation. However, the main disadvantages of SPE are high time consumption due to percolation of large volumes water samples and high cost since the SPE cartridges are manufactured to be used for single extraction.

The principle of SPE is the partition of analytes between a solid phase (SPE sorbent) and a liquid phase (sample). However, it is limited only for analytes that have a greater affinity for the SPE sorbent than for the sample matrix.

SPE procedure consists of four basic steps (Figure 8):

1) Conditioning and activation of SPE sorbent
2) Sample percolation when a sample is loaded on the SPE cartridge and analytes are retained
3) Washing step to remove interferences
4) Elution of analytes

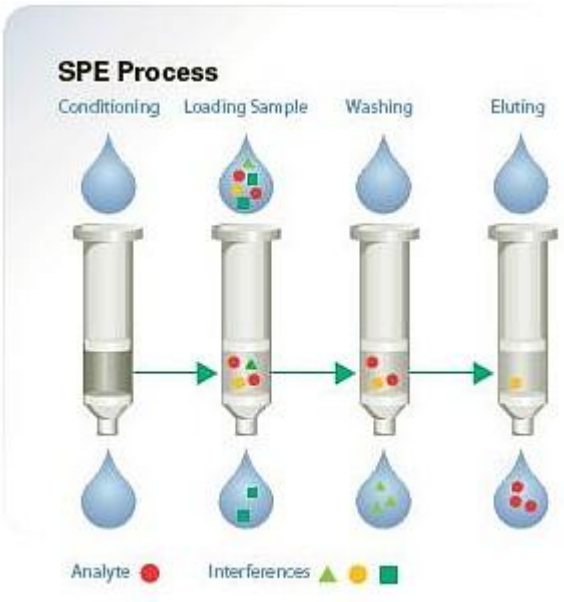


Figure 8: SPE procedure (adapted from [77]).

The choice of SPE sorbent is the key step in sample preparation as it influences the efficiency of SPE. Physico-chemical natures of analytes (e.g. polarity, pK_a values) are important properties as they highly influence further interaction with SPE sorbent. However, sample matrix and its interaction with SPE sorbent have to be taken in account as well. Nowadays, there is a wide variety of SPE sorbents available on market making easy the choice of the proper sorbent. The mostly used SPE sorbents are polymeric sorbents which improve the retention of polar compounds. These sorbents are suitable for multiclass analysis of pharmaceuticals in waters even without pH adjustment of sample. Among the mostly used SPE sorbent belongs copolymer of lipophilic divinylbenzene and hydrophilic N-vinylpyrrolidone known as Oasis HLB from Waters [75]. This sorbent allows working in wide range of pH (from pH 1 to 14). It does not contain free silanol groups to which may be bound many amphoteric pharmaceuticals Oasis HLB sorbent is used in for the extraction of analytes in multiresidue methods as well for analytes of the same properties. Mixed-mode sorbents based on Oasis HLB with ion-exchange groups (such as Oasis MCX and Oasis WCx containing strong and weak cation exchange groups, and Oasis MAX and WAX containing strong and weak anion exchange groups) become a valuable alternative [75][76].

Molecularly imprinted polymers (MIP) are a possibility for the extraction of polar analytes on the base of molecular recognition. These materials are not suitable for multiclass analysis. They may be used for extraction of analytes from very complex matrices with a minimum of co-extracted matrix interferences. However, there are limitations as they are available only for few classes of pharmaceuticals such as β-blockers, fluoroquinolones, antidepressants and non-steroidal anti-inflammatory drugs [76].

Solid-phase microextraction (SPME) is another option for the extraction of water samples in environmental analysis. It is based on the establishing a single partitioning equilibrium of analytes between the aqueous sample and solid sorbent [28]. In SPME, the fused-silica fiber with a solid stationary phase is used to collect analytes of interest. The analytes are sorbed to the solid phase which results in simultaneous extraction, clean-up and pre-concentration. After equilibration, the analytes are desorbed into an organic solvent followed by chromatographic analysis. The disadvantage of SPME might be the procedure development which requires the optimization of the equilibrium conditions for each compound. However, SPME showed some advantages over SPE such as decreased sample volume, ease and efficiency of extraction and better elimination of matrix effects [75]. Moreover, SPME might be coupled with LC-MS/MS system allowing automation of the extraction process and reduction of analysis time. In addition, accuracy, precision and sensitivity maintain using smaller amount of sample volume comparing to SPE [28].

3.2.2. Chromatographic analysis

The term "chromatography" originates at the beginning of the 20th century from Russian botanist Mikhail S. Tswett, who used a column packed with calcium carbonate and petroleum ether mobile phase to separate plant pigments (Figure 9). The name "chromatography" was derived from Greek words "*chroma*" which means color and "*graphy*" meaning writing.

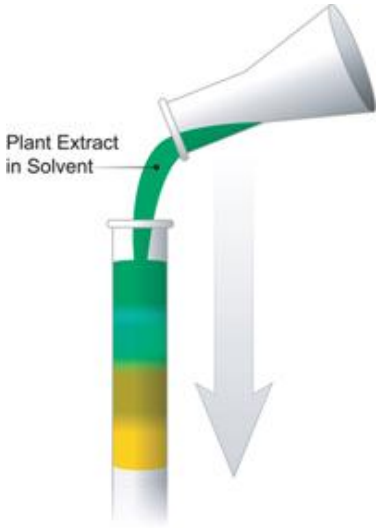


Figure 9: Tswett's experiment. He poured an extract of homogenized plant leaves into the column and allowed it pass through it. This was followed by pure solvent. During the passing of sample, different separated colored bands were observed (adapted from [78]).

However, Tswett's technique was ahead of his time and ignored for several decades before being reintroduced in 1941 by Martin and Synge, who were awarded the Nobel Prize in chemistry for the invention of partition chromatography in 1952. The development continues rapidly to possibility to switch the instruments based on atmospheric pressure and gravity flow for the pressurized pumping systems, for which a new term high-pressure liquid chromatography originates. Later, high-performance liquid chromatography was invented and HPLC acronym used.

Nowadays, liquid chromatography (LC) is the most widely used separation method because of its advantage to separate analytes in mixtures and simultaneously perform their qualitative and quantitative analysis. The function of chromatography is the separation of a mixture of compounds into individual components. Briefly, when the sample is injected onto LC system, it moves with the mobile phase flow and the separation of analytes occurs as a partition between the mobile phase and stationary phase. Compounds whose distribution ratio is higher for stationary phase moves slowly and require a longer time to pass through the column and as a result they are separated from the fast-moving components which have less affinity for the stationary phase.

Consequently, analytes with similar distribution ratios can be separated using a suitable mobile phase, stationary phase and sufficient time.

High-performance liquid chromatography (HPLC) is the most used separation technique in many laboratories. Basically, an HPLC system consists of pumping system, injector, column, detector and computer data station (Figure 10). The heart of HPLC system is the column where the actual separation occurs. A mobile phase is pumped from a reservoir, through an injector, into the analytical column, and to the detector. A sample dissolved in the mobile phase or in a similar solvent is injected into the flowing mobile phase on the column. Separation which occurs is specific for the type of column and separated peak elutes into the detector where it causes a signal to be sent to the data system.

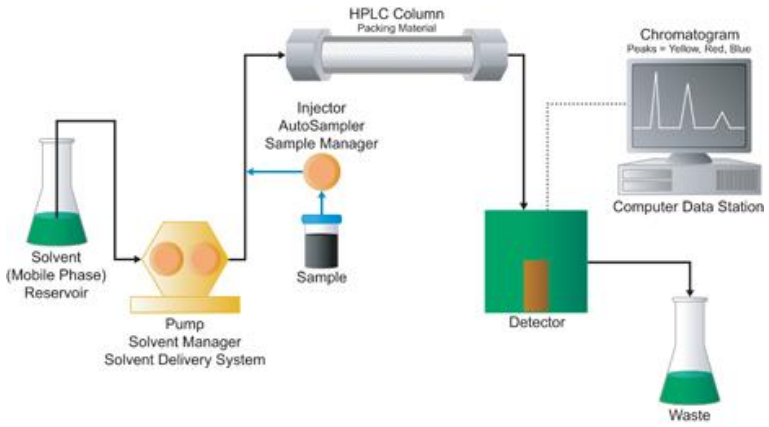


Figure 10: A schematic of HPLC system (adapted from [79]).

Conventional HPLC instruments can operate at back-pressures up to 42 MPa. Traditionally, the analytical columns used in HPLC were of 4.6 mm internal diameter from 150 up to 250 mm long using silica beads typically ranging between 3- to 5- or 10-μm particle diameter. However, the necessity for fast analysis leads to employment of short columns (50 – 100 mm long) with small particles and narrow diameter (2.5 mm). Nowadays, silica-organic hybrid stationary phases generally consist of porous particles (sizes bellow 2 μm) providing a large surface area. Using these sub-2-μm particles leads to the development of ultra-high performance liquid chromatography (UHPLC). The first UHPLC system was introduced in 2004 by Waters Corporation. After 2004 a huge development of UHPLC techniques happen and nowadays a lot of manufactures sell different UHPLC systems.

3.2.2.1. Basic terms of liquid chromatography

A chromatogram is a graphical representation of detector response, eluent concentration or other quantity used as a measure of analyte concentration, versus time,

volume or distance. Idealised chromatograms are represented as a sequence of Gaussian peaks on a baseline [80]. The peak of the compound can be described by retention time (t_r) as qualitative index, by the peak area (A) or by peak height (h) as quantitative indexes and by the peak width at half-height (w_h) or the peak width between the points of inflection (w_i) (Figure 11):

The performance of a chromatographic system is described by following parameters: capacity factor, selectivity, plate number and resolution which will be described later.

In Gaussian peaks there is the relationship:

$$w_h = 1.18\, w_i \qquad \text{eq. 8}$$

w_h = peak width at half height
w_i = peak width between the points of inflection

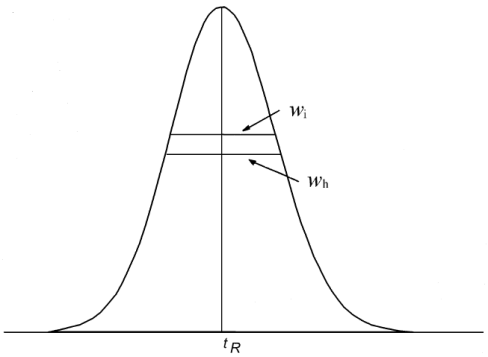


Figure 11: An illustration of basic characteristics of chromatographic peak [81].

Retention data

- The retention time (t_R) of an analyte is the time between the injection of the solute onto chromatographic system and the time it appears at highest concentration (peak maximum) in the eluate passing through the detector. The identification of analytes using LC is based on the comparison of the retention time of analyte with the retention time of relevant standard.

- The retention volume (V_r) may be calculated from the retention time as follows:

$$V_r = t_r\, v \qquad \text{eq. 9}$$

t_r = retention time of compound
v = the flow rate of mobile phase

- The *capacity factor (k')* (also known as the mass distribution ratio or retention factor) describes how strongly a compound is retained by the stationary phase. It is defined as the ratio of the time a solute spends in the stationary phase versus the time it spends in the mobile phase. The higher value of k' the more is the compound retained on the column, eluted later and separated from the time. It can be expressed by the equation:

$$k' = \frac{amount\ of\ solute\ in\ stationary\ phase}{amount\ of\ solute\ in\ mobile\ phase} = K_c \frac{V_S}{V_M} \qquad \text{eq. 10}$$

K_c = equilibrium distribution coefficient (also known as distribution constant)
V_S = volume of the stationary phase
V_M = volume of the mobile phase

The capacity factor may be determined from the chromatogram using the expression:

$$k' = \frac{t_r - t_m}{t_m} \qquad \text{eq. 11}$$

t_r = the retention time (or volume)
t_m = elution time of the nonretained compound

- The relative retention (*r*) is calculated from the expression:

$$r = \frac{t_{r2} - t_m}{t_{r1} - t_m} \qquad \text{eq. 12}$$

t_{r2} = the retention time of the peak of interest
t_{r1} = the retention time of the reference peak
t_m = elution time of the nonretained compound

Chromatographic data

- The symmetry factor (A_s) or tailing factor represents the symmetry of the chromatographic peak. The symmetry of the peak is important from the point of view integration of the peak and the quantitation. It can be expressed by the equation:

$$A_s = \frac{w_{0.05}}{2d} \qquad \text{eq. 13}$$

$w_{0.05}$ = the peak width at one-twentieth of its height
d = the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height – Figure 12

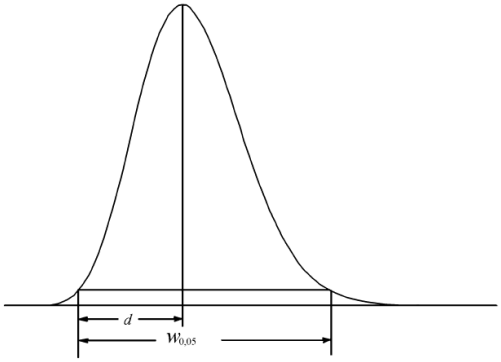


Figure 12: The determination of the symmetry factor [81].

The value 1.0 signifies the peak is ideally symmetric. Higher value expresses tailing of the peak and lower value than 1.0 means fronting of the peak. The accepted values by pharmacopoeias are in range 0.8 – 1.5 [80].

- Column performance and apparent number of theoretical plates

The column performance (apparent efficiency) may be expressed as the apparent number of theoretical plates (N), series of small zones or plates where partitioning between mobile phase and stationary phase occurs. It may be calculated from data obtained under isothermal, isocratic or isodense conditions, depending on the technique. It can be expressed by the equation where the values of t_r and w_h have to be expressed in the same units (time, volume or distance):

$$N = 16\left(\frac{t_r}{w}\right)^2 = 5.54\left(\frac{t_r}{w_h}\right)^2 \qquad \text{eq. 14}$$

t_r = the retention time of the compound
w_h = the peak width at half-peak height

The apparent number of theoretical plates of a column is dimensionless parameter; it is not fixed and varies with the component as well as with the column (length, diameter and particle size) and the retention time. Higher value of number of theoretical plates means higher efficiency of the analytical column. However, by the reason the parameter N is highly influenced by the length of the column, it is better to use the height equivalent to a theoretical plate (HETP) parameter for the comparison on analytical columns.

- The height equivalent to a theoretical plate (HETP) [μm] is defined as the length of column (l) that participates in one mass transfer equilibrium between stationary phase and the mobile phase. It can be calculated by the equation:

$$HETP = \frac{l}{N} \qquad \text{eq. 15}$$

N = number of theoretical plates
l = the column length [μm]

The relation of plate height HETP and number of theoretical plates N is non-proportional. It means that lower values of plate height (HETP) means higher efficiency of the analytical column.

Separation data

- The *selectivity factor (α)* defines the extent of separation between two components and it is given by the ratio of their capacity factors. It can be expressed as the ratio of retention times (volumes) of two peaks. It is showing the column's relative selectivity for two components and it can be expressed by the equation:

$$\alpha = \frac{k'_B}{k'_A} = \frac{t_{rB} - t_m}{t_{rA} - t_m} \qquad \text{eq. 16}$$

k'_A, k'_B = capacity factors of two peaks
t_{rA}, t_{rB} = retention times of compounds
t_m = elution time of the nonretained compound
B = refers to a late-eluting component ($t_{rB} > t_{rA}$)

- The separation of two chromatographic peaks A and B can be expressed in terms of the *resolution (R)* which is a quantitative measure of the degree of the separation between two compounds with peaks of similar height. It is given by the equation:

$$R = \frac{1.18\ (t_{rB} - t_{rA})}{w_{hA} + w_{hB}} \qquad \text{eq. 17}$$

t_{rA}, t_{rB} = retention times
w_{hA}, w_{hB} = the peak widths at half-height

The resolution of 1.5 corresponds to almost baseline separation of two equally large peaks. This value is accepted by the authorities for fulfill the requirements of system suitability test [80][81].

Another possibility for counting the resolution is by using the three main parameters of chromatographic separation: selectivity, capacity and efficiency (eq. 18).

$$R = \frac{1}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k\prime}{k'+1}\right)\sqrt{N} \qquad \text{eq. 18}$$

α = selectivity factor
k' = capacity factor
N = number of theoretical plates

From this equation it is possible to see how each parameter can influence the chromatographic separation:

- selectivity factor – the change of stationary phase, change of mobile phase, change of the flow rate velocity
- capacity factor – the amount of stationary phase in analytical column, the change of stationary or mobile phase, the change of temperature
- efficiency – the flow rate of mobile phase, length of column, the size of sorbent particles, temperature, viscosity

Precision of quantification

The signal-to-noise ratio (S/N) influences the precision of quantification. It is mainly used for the calculation of sensitivity of chromatographic system such as limits of detection and quantitation. It is expressed by the equation:

$$S/N = \frac{2H}{h} \qquad \text{eq. 19}$$

H = height of the peak corresponding to the component concerned, in chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20times the width at half-height

h = range of the background noise in a chromatogram after injection or application of blank, observed over a distance equal to 20 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found (Figure 13)

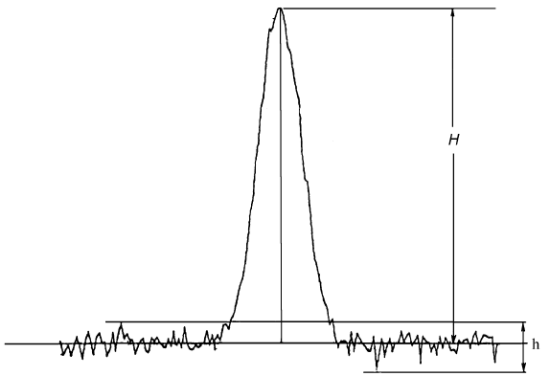


Figure 13: The determination of signal-to-noise ratio [81].

3.2.2.2. Ultra High Performance Liquid Chromatography (UHPLC)

Current trends in analytical laboratories are miniaturizing of equipment, decreasing the analysis time and lowering solvent consumption. The development in fast LC has been primarily related to UHPLC, monolithic columns, fused core columns, and high temperature LC.

Monolithic columns provide one approach to fast LC separations without compromising efficiency or resolution. Monolithic columns are characterized by a bimodal pore size distribution due to a sol-gel technology, which allows the formation of macropores and mesopores in their structure. This high porosity of monolithic columns permits operation at high flow rates (up to 10 mL/min) on relatively long columns without a high increase in back-pressure. This allows use of conventional LC systems for high-speed and high-efficiency separations. Another advantage of monolithic columns is the short time which they need to equilibrate, and the possibility to couple several columns to increase the column efficiency, and the reduction in analysis time. However, a disadvantage of monolithic columns is the limited number of variety of stationary phases (e.g. silica gel, C_{18}, C_8) and internal diameters for a maximum column's length of 100 mm that are commercially available which limits their practical application.

Fused core columns may increase the separation efficiency and speed of analysis using superficially porous particles. These particles are characterized by a 1.7 μm fused core and 0.5 μm layer of porous silica coating, resulting in a total particle diameter of 2.7 μm. Fused core silica columns shown improvement in chromatographic separation over fully porous particles in reversed phase. Similar results were obtained from the comparison of fused core columns and sub-2-microne columns used in UHPLC. However the main advantage of using fused core columns is the efficiency in high speed analysis without the generation of high-back pressures, which allows using these columns in HPLC systems [83].

The use of high column temperatures can improve separation speed and column efficiency. Low viscosity and high diffusivity of a mobile phase at high temperatures produce much lower mass transfer resistance. The reduced mobile phase viscosity and consequent enhanced diffusivity allow for operation at high flow velocity and permit use of longer columns or smaller particles, which can increase efficiency and speed, respectively [82].

However, the introduction of UHPLC instrument in 2004 by Waters Corporation led to revolution in the analytical laboratories. UHPLC is a separation technique using columns packed with sub-2-μm porous particles and capable of operating at backpressures up to 15 000 psi (approx. 100 MPa or 1000 bars). This leads to the major advantages of UHPLC over conventional HPLC as is high separation efficiency and

resolution, high sensitivity, shorter retention times (faster analysis) and much lower solvent consumption.

It is known that the most effective way how to obtain fast and highly efficient separations is reducing the diameter of column particles, as is shown in van Deemter theory. Based on this theory, efficiency is expressed as the plate height (H) which is sum of three constants:

$$H = A + B + C \qquad \text{eq. 20}$$

H = plate height
A = constant related to eddy diffusion
B = constant related to longitudinal diffusion
C = mass transfer in mobile phase and stationary phase

The theory describes the relationship between linear velocity (flow rate) and plate height (H or column efficiency). Since particle size is one of the variables, a van Deemter curve can be used to investigate chromatographic performance. When the plot plate height (H) versus linear velocity (u) (van Deemter plot) is traced for different particles diameter (Figure 14), it is possible to see when the particle size decreases to less than 3 µm, there is a significant gain in efficiency, but also the efficiency does not diminish at increased flow rates or linear velocities.

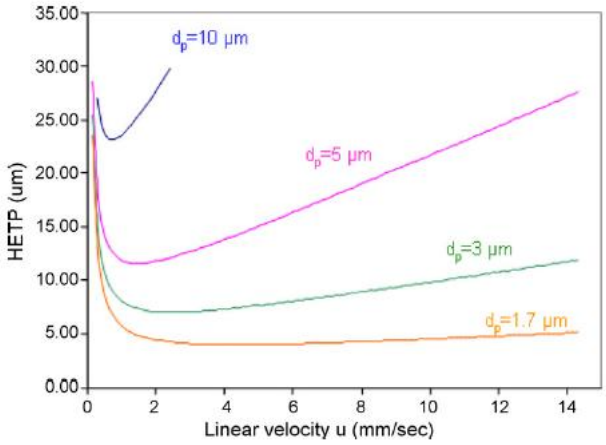


Figure 14: *Van Deemter* curves for different particle sizes (10, 5, 3, 1.7 µm) [83].

UHPLC instrumentation

Using sub-2-µm particles with high linear velocities lead to an increase of back-pressure in the LC system. However, this pressure is not compatible with conventional HPLC systems, since they are not able to work at pressures upper than 400 bar (6000 psi). Moreover, due to the high backpressure that has to support and to operate in fast mode with columns with low internal diameter (e.g. 2.1 mm), UHPLC systems have some

differences relatively to conventional HPLC systems, that allows to limit the frictional heating and reduce the solvents consumption. These systems must to fulfill some requirements on hardware, such as pumps, valves, injector, connecting tube, columns, and detectors.

Figure 15: Acquity UPLC system from Waters Corporation [84].

UHPLC system consists of the same important parts as conventional HPLC (Figure 15) such as pump system, injector, column thermostat and detector. However, some changes in construction have to be done in order to achieve high efficiency of the system. Commercially available UHPLC systems are equipped with pump systems very similar to those of conventional HPLC, but the main difference is that UHPLC pumps are able to withstand high pressures and have smaller system volumes due to a small mixer. Moreover, a small flow cell and connecting tubes with a small internal diameter are used [82]. The most challenging was the sample introduction modification as it was necessary to construct a valve that seals at high pressures and deliverer small volumes of sample into a column.

UHPLC produces narrow peaks. For that a detector with a small detection volume and fast acquisition rate in order to ensure high efficiency is required. Most of the commercial UHPLC systems are equipped with a modified UV detector that has a flow cell with a much smaller volume (typically 0.5-2.0 μL) compared to conventional HPLC systems, in order to minimize the extra-column volume. According to Lambert-Beer's law the signal depends on light path-length. However, small flow cells imply a small path-length. To solve this problem and maintain the path-length, conventional flow cell SS cylinder were replaced by light guided flow cells, which can be made from Teflon or fused silica capillaries and prolong the path-length of light transmission based on the difference of refractive indexes of fluid and cladding [82]. At the same time, the detection software has to be capable of achieving a fast detector time constant (<0.1 s) and a high data acquisition rate in order to obtain enough data points for a narrow peak (typically > 20 Hz) [82].

Stationary phases

The development of UHPLC has been connected with the development of stationary phases. To obtain an efficient separation, the columns have to be packed with mechanically and chemically stable stationary phases. The stationary phases have to be able to support high backpressures and a wide pH range. Most of the commercially available columns for UHPLC are made of porous particles. They are typically of a length of 10 cm and they are packed with 1.7 to 2.0 µm particles, which produce pressures compatible with the characteristics of the UHPLC systems. Typically, UHPLC columns have an internal diameter of 1.0 or 2.1 mm, in order to minimize the frictional heating effect and assure a good sample loading capacity. The internal diameter has also the advantage of allowing a significant reduction in solvent consumption [82]. Nowadays there are several stationary phases available for use in UHPLC (Figure 16). These may include both silica and hybrid materials, and may also be modified with different chemistries, such as C_8, C_{18}, Phenyl, Cyano and Shield RP18, among others. The hybrid stationary phases have the advantage of being chemically stable in a pH range between 1 and 12.

Column name	Stationary phase support	Particle size [µm]	Limitations			Manufacturer
			pH	Column chemistry	Temperature	
Acquity BEH	Hybrid	1.7	1–12 2–11 1–8 2–11	(C8, C18, Phenyl) (Shield) (Silica for HILIC) (Amide, Glycan—HILIC phases)	20–90	Waters
Acquity HSS	Silica	1.8	2–8 1–8	(T3, C18 SB) (C18)	20–45	Waters
Alltima HP	Hybrid	1.5	1–10	(HILIC)	20–60	Alltech
Platinum	Silica	1.5	2–8	(C8, C18)	20–60	Alltech
GP Series	Silica	1.8	2–8.5	(C8, C18, C4)	20–60	Sepax
HP Series	Silica	1.8	2–8.5	(PHE, CN, NH_2 SCX, SAX, silica, HILIC)	20–60	Sepax
Poly-RP	Hybrid (PS/DVB)	1.0/1.7	1–14	(PHE)		
Hypersil Gold	Silica	1.9	1–11 2–9 2–8	(C18) (C8, Q) (PFP)	25–60	ThermoElectron
Nucleodur	Silica	1.8	1–11 1–10 1–9	(C8,C18) (C18 isis, Sphinx RP) (C18 pyramid)	Up to 85	Machery Nagel
Pathfinder	Hybrid	1.5	1–12	(AS, AP, PS, MR)	Up to 250	Shimadzu
Pinnacle DB	Silica	1.9	2.5–7.5	(C18, PFP-propyl, silica, acqueous C18, CN, C8, PAH, X3-C18)	Up to 80	Restek
Pronto Pearl		1.8 totally porous 1.5 nonporous	2–8	(C18, C8, aminopropyl) (C18)	20–60	Bischoff
TSKgel Super ODS	Silica	2.0	2.0–7.5	(C18, C8, PHE)	20–60	Tosoh
YMC ultra-fast	Silica	2.0	2–8	(C18, Hydro C18)	20–60	YMC
Zorbax	Silica	1.8	2–9 2–8 1–6 2–11.6	(Eclipse plus C8, C18, Eclipse XDB-C18, C8, PHE) (Eclipse phenyl-hexyl, PAH, Elipse XDB-CN) (StableBond) (Extend-C18)	Up to 60 80–100 StableBond	Agilent

Figure 16: An overview of currently available sub-2-microne analytical columns and their properties (adapted from [83]).

3.2.3. Mass spectrometry

The increase of importance of mass spectrometry (MS) during last years is unquestionable. Nowadays MS is an essential analytical tool in physics, chemistry, biochemistry, pharmacy, medicine and environmental sciences. Mass spectrometry has developed and changed tremendously during the 20th century and still is changing. A large number of mass spectrometers have been developed since the first Thomson's experiments in 1897 until A.A.Makarov who has described a new type of mass analyzer Orbitrap in 1999 [85].

The unique capabilities of mass spectrometry result in its wide popularity. MS is able to measure accurate molecular mass and to provide information on structurally diagnostic fragment ions of an analyte. The high sensitivity of MS was demonstrated by detection of molecules in attomole and zeptomole amounts. MS is applicable to all types of samples: volatile and nonvolatile; polar or nonpolar; solid, liquid, or gaseous materials and it can be connected with high-resolution separation systems [86].

The basic principle of mass spectrometry is to convert molecules or atoms into gas-phase ionic species, to separate these molecular ions and their charged fragments on the basis of their *mass-to-charge ratio (m/z)* and to detect them qualitatively and quantitatively by their *m/z* and abundance and to display it in the form of mass spectrum [86]. A mass spectrometer consists of three basic parts: an ion source, a mass analyzer and a detector. The general scheme of a modern mass spectrometer is shown (Figure 17).

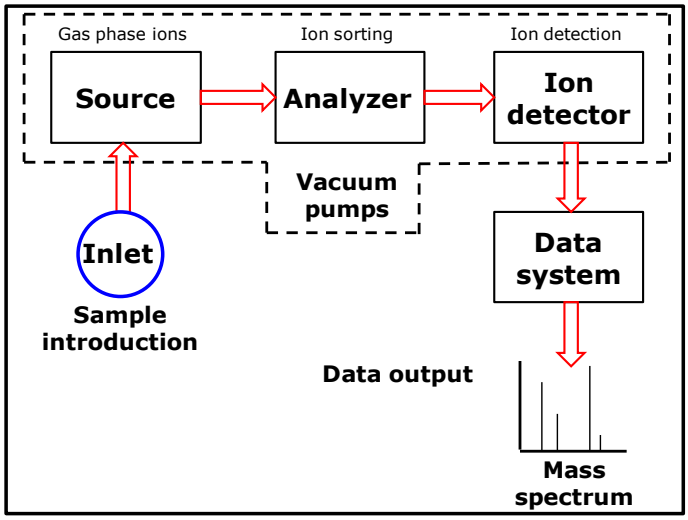


Figure 17: A scheme of mass spectrometer (adapted from [87]).

An inlet system transfers a sample into the ion source. Ion source converts the neutral sample molecules into gas-phase ions and a mass analyzer separates and mass-analyzes the ions. Further detector measures and amplifies the ion current of mass-resolved ions. The ionization and separation steps are carried out under high vacuum

which prevent the collision of ions with residual gas molecules during their flight from the ion source to the detector. Collisions may lead to fragmentation of the molecular ions and may also produce a different species through ion-molecule reactions. These processes will consequently reduce sensitivity, increase ambiguity in the measurement, and decrease resolution [86]. For these reasons, mass spectrometers are often provided by rotary vacuum pumps in combination with turbo-molecular pumps allowing achieving vacuum typically 10^{-3} - 10^{-10} Pa. MS systems are controlled by a computer, which also records, processes, stores and displays acquired MS data [86].

3.2.3.1. Ion sources and modes of ionization

The ionization of the analyte is the first essential and crucial step in MS analysis. The main function of the ion source is to introduce sample molecules or atoms into the mass spectrometer and convert them to charged or ionized forms. This step requires the removal or addition of an electron or proton(s). Moreover the excess of energy transferred during ionization may break the molecule into characteristic fragments. The ion source should have the following desirable characteristics: high ionization efficiency, a stable ion beam, a low-energy spread in the secondary-ion beam, minimum background ion current and a minimum cross-contamination between successive samples [86]. There is a wide variety of ionization techniques and ion sources. Their classification could be done from different points of view. Some ionization techniques are soft (atmospheric pressure chemical ionization (APCI), atmospheric pressure photo ionization (APPI), electrospray ionization (ESI), chemical ionization (CI) and matrix-assisted laser desorption/ionization (MALDI)). A proton is added or subtracted to yield $[M+H]^+$ or $[M-H]^-$ ions, respectively. In contrast, some ionization techniques are very energetic which cause extensive fragmentation (electron ionization (EI)). Ionization is performed by ejection or capture of an electron by an analyte to produce a radical cation $[M^{+\bullet}]$ or anion $[M^{-\bullet}]$, respectively. Another point of view is operation of an ionization source. Some sources require vacuum (EI, MALDI) and others can operate at atmospheric pressure (APCI, APPI, ESI) which allows the combination of MS with separation techniques such as LC or capillary electrophoresis (CE). Such techniques are called “hyphenated”, e.g. LC-MS. The introduction of atmospheric pressure ionization (API) techniques enables to analyze a large number of compounds that can be successfully analyzed by LC-MS. During API, the analyte molecules are ionized first, at atmospheric pressure and then analyte ions are mechanically and electrostatically separated from neutral molecules. Common atmospheric pressure ionization techniques are ESI, APCI and APPI.

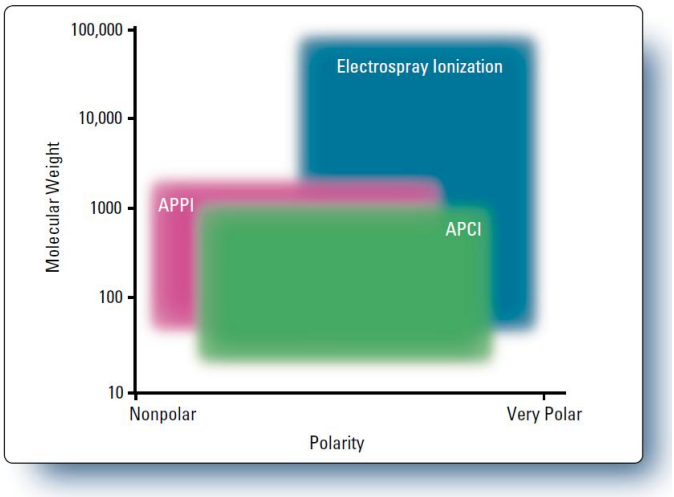


Figure 18: Applications of various LC/MS ionization techniques [88].

The choice of an ionization technique is dictated largely by the nature of the sample. The polarity and molecular weight of analyte (Figure 18) are essential analyte properties.

Electrospray ionization (ESI)

This mode of ionization represents a breakthrough in the analysis of large biomolecules. Moreover, it has also become the most successful interface for LC/MS and CE/MS applications. The development of ESI culminated in 2002 when John Fenn was awarded by Nobel Prize in Chemistry. ESI is recommended for use with highly polar and ionized compounds. It is a very soft technique that results in little fragmentation.

ESI is accomplished by passing an LC eluent down through a heated metal capillary tube along which a 3 to 4 kV electric charge differential is applied. ESI is a process that produces a fine spray of highly charged droplets under the influence of an intensive electric field. Those charged droplets are converted into gas-phase ions by evaporation of the solvent which is assisted by a flow of hot nitrogen.

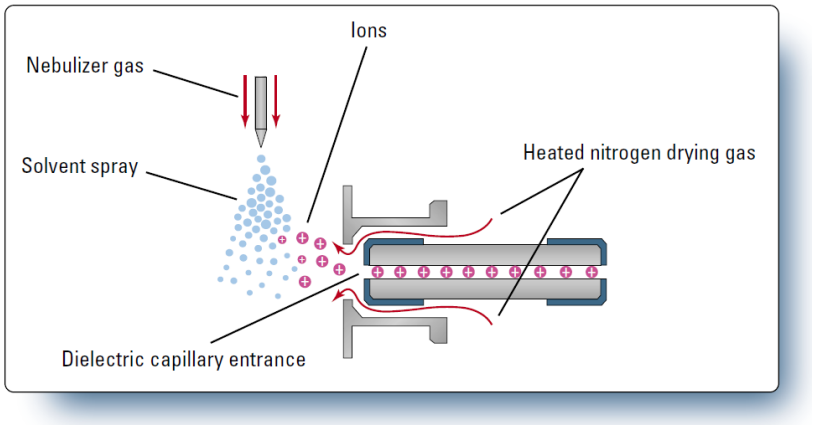


Figure 19: A schematic of electrospray ion source [88].

During the process of droplet evaporation, some of the dissolved ions are released into the atmosphere (Figure 20). This phenomenon is known as Coulomb explosion. The resulting ions are transported from the atmospheric-pressure region to the high vacuum of the mass analyzer via a series of pressure-reduction stages.

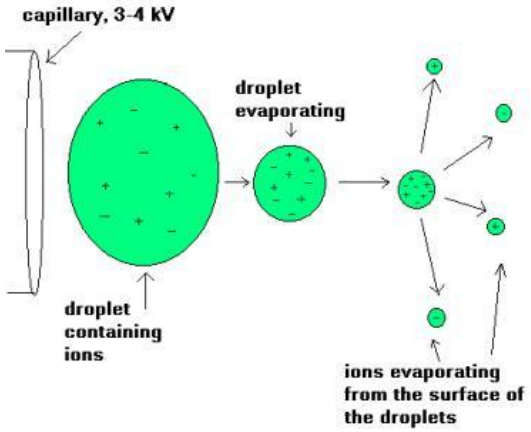


Figure 20 : Coulomb explosion [89].

ESI analysis can be performed in positive and negative ionization modes. The polarity of ions to be analyzed is selected by the capillary voltage bias. A novel feature of the ESI mass spectrum is the formation of intact molecular ions of the analyte. Fragmentation, if desired, can be induced in ESI source by increasing the sampling cone voltage. This process is known as *in-source collision-induced dissociation* (CID).

The coupling of an ESI source with a quadrupole mass analyser is the most successful ESI-MS combination [86]. However, there are combination of ESI with time-of-flight (TOF), quadrupole ion trap (QIT), linear ion trap (LIT), ESI-Orbitrap and Fourier transformation-ion cyclotron resonance (FT-ICR).

3.2.3.2. Mass analyzers

The mass analyzer is the heart of a mass spectrometer. The performance of mass spectrophotometer largely depends on the design of mass analyzer and associated ion optics. The role of mass analyzers is to separate ions formed in the ion source according to their *mass-to-charge ratio (m/z)* through the use of electric and/or magnetic fields and to focus all mass-resolved ions to the detector. In addition, a mass analyser maximizes the transmission of all ions that enter from the ion source. A moving charged particle can be distinguished from another ion on the basis of differences in their momentum, kinetic energy, and velocity. A mass analyzer makes use of one or more of these properties to mass-resolve and to focus various ions. Mass analyzers can be divided into categories according to their separation principle such as magnetic sector, quadrupole, QIT, LIT, orbitrap, TOF, and ion cyclotron resonance (ICR).

The performance of a mass analyser is evaluated on the basis of the following desirable features: mass range, resolution, efficiency, mass accuracy, linear dynamic range, speed, sensitivity and adaptability. Tandem MS (MS/MS) capability, small size, and lower cost are other desirable characteristics of a mass analyzer

High resolution is a desirable feature of an MS, as it helps to perform accurate mass measurements, to resolve isotopically labeled species when the percent incorporation of the label is to be determined, to resolve an isotopic cluster when the charge state of high-mass compounds is to be determined, to enhance the accuracy of quantification, and to unambiguously mass-select precursor ions in MS/MS experiments [86].

Quadrupole MS

Quadrupole instruments are the most widely used type of MS. A quadrupole is constructed from four precisely matched parallel metal rods. The mass separation is accomplished by the stable vibratory motion of ions in a high-frequency oscillating electric field that is created by applying direct-current (DC) and radio-frequency (RF) potentials to these electrodes. Under a set of defined DC and RF potentials, ions of a specific *m/z* value pass through the geometry of quadrupole rods [86].

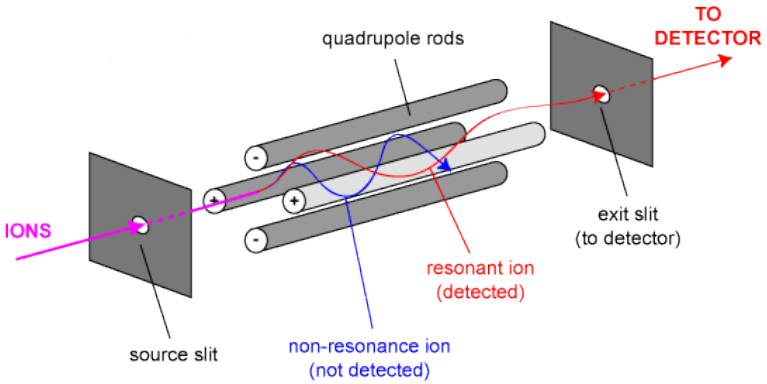


Figure 21: A schematic of quadrupole mass analyzer [90].

3.2.3.3. Detectors

The role of detector is to convert the beam of mass-resolved ions into an electrical signal which is further amplified, stored and displayed in the form of mass spectrum. Sensitivity, accuracy, resolution, response time, stability, wide dynamic range, and low noise are the most important characteristics that are sought in any ion detector [86]. The most used detectors are electron multipliers.

3.2.3.4. Tandem MS (MS/MS)

MS/MS has achieved an enviable status as an analytical tool to identify and quantify compounds in complex mixtures. MS/MS refers to the coupling of two stages of mass analysis, either in time or space. Of all ionization techniques, only EI provides abundant structural information. In order to obtain additional structure-specific information by other ionization techniques, it has become essential to perform MS/MS experiments [86].

The concept of tandem MS is based on that the system involves two mass spectrometry systems (Figure 22). The first MS system (MS1) performs the mass selection of a desired target ion from a stream of ions produced in the ion source. This mass-selected ion undergoes either unimolecular fragmentation or a chemical reaction in the intermediate region. The second MS (MS2) performs the mass analysis of the product ions that are formed in the intermediate step. Basically, the first stage of MS/MS separates a mixture of ions into individual components, and the second stage act as an identification system for the mass-resolved ions. Tandem MS is not restricted only to two stages of mass analysis (MS/MS) but it is also possible to perform multistage MS experiments (MS^n). Multistage MS experiments are mostly performed with ion-trap instruments [86].

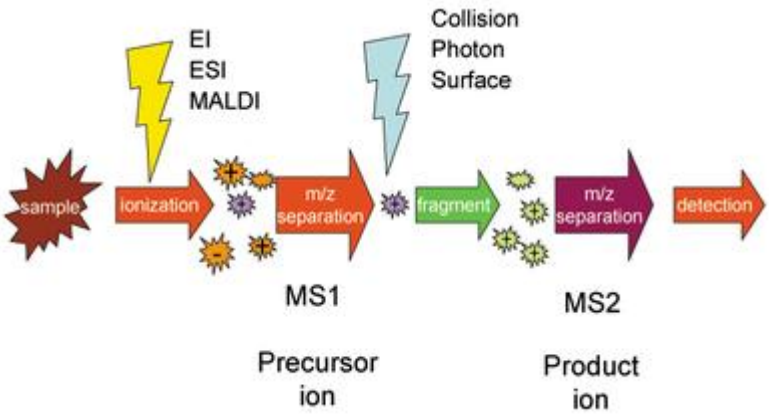


Figure 22: A schematic of tandem mass spectrometry [91]

Tandem MS/MS instruments

Tandem MS instruments are classified into two categories: tandem in-space and tandem in-time. For tandem in-space instruments it is characteristic that the three steps of MS/MS (e.g mass selection, fragmentation and mass analysis) are carried out in three discrete regions. This is achieved by arranging two mass spectrometers sequentially. Triple quadrupole, TOF-based and magnetic instruments are example of tandem in-space instruments. For tandem in-time instruments it is characteristic that the three steps of MS/MS are performed at the same region but by employing a temporal sequence. LIT, QIT and FT-ICR can be mentioned as an example of this category of tandem MS instruments.

Tandem mass spectrometry in triple quadrupole instruments

The MS/MS efficiency of modern QqQ instruments is very high. In addition they have the advantages of low cost, operational simplicity, straightforward scan laws and a linear mass scale. They have unmatched detection sensitivity in the SRM mode and quantification [86].

In QqQ instruments, three quadrupoles are arranged sequentially. The first (Q_1) and last (Q_3) quadrupoles operate as normal mass filters and the middle one as RF-only quadrupole (Q_2). The DC and RF potentials both control the operation of Q_1 and Q_3, whereas Q_2 is operated with only the RF potential. The RF-only mode of operation of Q_2 allows all ions to pass through it. The Q_2 also serves as a total ion containment region and a collision cell. However, some modern contemporary instruments contain an octopole or hexapole collision cell instead of conventional quadrupole cell. Because ions in the range 0 to 100 eV can be transmitted through quadrupoles, the MS/MS spectra obtained with triple-quadrupole instruments contains ions formed via low-energy fragmentation processes. The collision energy can be controlled by offsetting the voltage between the ion source and Q_2. All four types of scan modes can be performed in QqQ instruments.

Types of scan functions

MS/MS provide data acquired in the following four scan modes:

- The *product-ion scan* (Figure 23) is the most useful mode of MS/MS in the structure elucidation of a specified analyte. It is widely employed with ESI ionization and/or LC-MS. To acquire this spectrum, the first mass analyser is set to transmit only the precursor ion chosen into collision cell where it interacts with a collision gas and fragments. The MS2 is scanning over a required *m/z* range. Therefore the final spectrum contains only product ions that are formed exclusively from a mass-selected precursor ion.

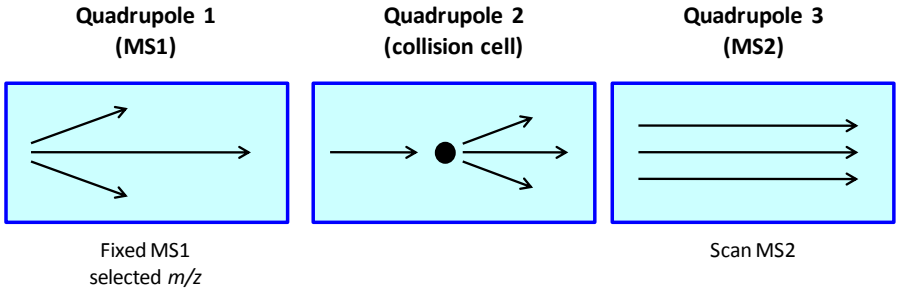


Figure 23: A scheme of product-ion scan.

- The *precursor-ion scan* (Figure 24) provides a spectrum of all precursor ions that might fragment to a common, diagnostic product ion. To acquire this spectrum, the MS2 is

adjusted to transmit a chosen product ion and the MS1 is scanning over a certain *m/z* range to transmit only those precursor ions that fragment yields the chosen product ion. This scan is useful for the identification of a closely related class of compounds in a mixture.

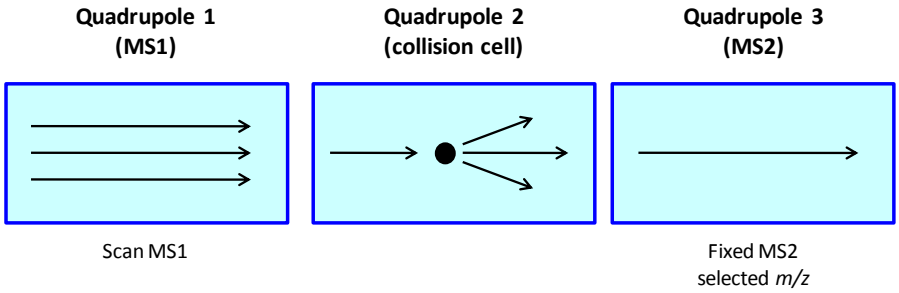


Figure 24: A scheme of precursor-ion scan.

- The *constant-neutral-loss scan* (Figure 25) monitors all precursors that undergo the loss of a specified common neutral. To acquire this information, both mass analysers are scanned simultaneously, but with a mass offset that correlates with the mass of the specified neutral. It is useful in the selective identification of closely related class of compounds in a mixture.

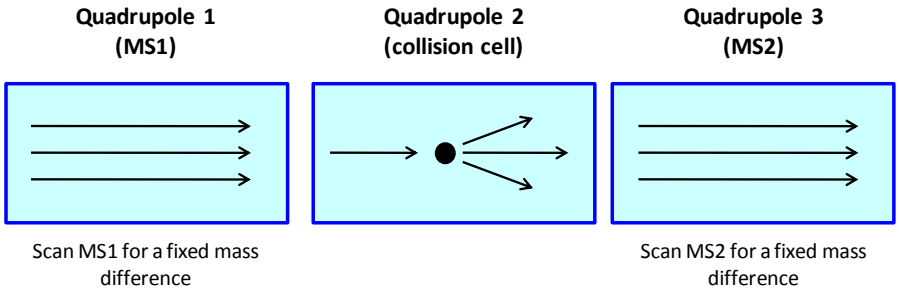


Figure 25: A scheme of constant-neutral-loss scan.

- The *selected-reaction monitoring* (SRM) (Figure 26), is the most used mode in quantitative measurements of analytes present in complex mixtures. To obtain this information, both mass analysers are adjusted to monitor one or more chosen precursor-product pairs of the analyte.

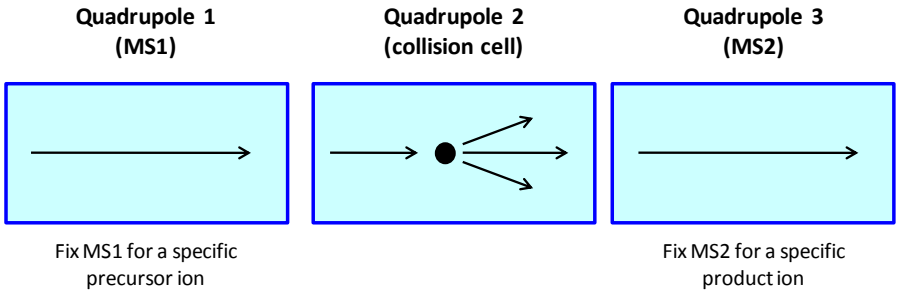


Figure 26: A scheme of selected-reaction monitoring mode.

3.2.3.5. Hyphenated separation techniques

The coupling of LC with MS was a result of the requirement to obtain more information from one analysis of real-world samples such as environmental and biological samples. These samples are very complex matrices containing not only measured analytes but also other interfering compounds. A range of separation techniques such as gas chromatography (GC), HPLC, CE and also thin-layer chromatography has been combined with MS. The role of separation techniques in the separation of complex mixtures is unquestionable. Similarly, MS has an indisputable position in analytical chemistry as a highly structure-specific technique that can provide structural identity of a wide range of compounds. The connection of LC with MS enables the separation and identification of complex mixture in one analysis. At present LC-MS is one of the most sensitive analytical methods with the high power of mass separation, and very good selectivity can be obtained.

A connection of LC-MS system consists of three major components: a separation device, an interface and a mass spectrometer (Figure 27). The purpose of an interface is to transport the separated components into the ion source of an MS for their identification. An ideal interface should not affect the performance capabilities of either the chromatography or the MS system. The interface must remove most of the mobile phase (volatile solvents and its additives) without removing the target compounds of interest. The second function of the interface is to ionize the target compounds, since many of the components in LC eluent are uncharged, transferred ions into MS system and not degrade the vacuum of the MS system.

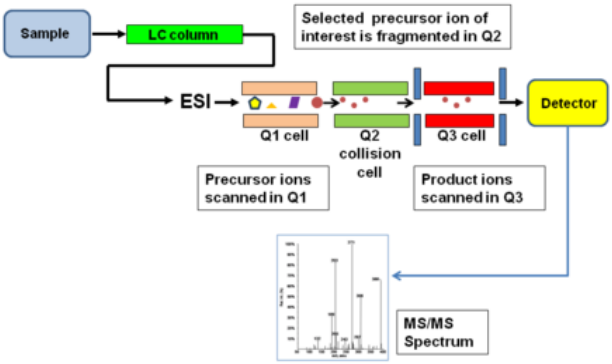


Figure 27: A schematic of LC-MS with ESI source (adapted from [92]).

Electrospray ionization interface

ESI is the widely used format of an atmospheric-pressure ionization source. It has four main advantages enabling its used in variety areas of research:

1) the potential for the analysis of a variety of non-volatile and thermally labile molecules of low to very high molecular mass
2) ionization occurs at atmospheric pressure
3) it is compatible with reverse-phase LC solvents
4) a range of solvent flow can be accepted

The composition and flow rate of mobile phase is the most important parameter in LC-MS. The flow rate determines the efficiency of ESI such as size and distribution of droplets forms during ESI. At high flow rates the formation of droplets is not stable because of formation of large droplets which lead to electric breakdown. Similarly the type of solvent in mobile phase determines the efficiency of ionization. Polar solvents such as methanol, acetonitrile are suitable for ESI. However, water as fluid with high surface tension is difficult to electrospray. For this reason, volatile additives such as formic acid, acetic acid, ammonium acetate, ammonia are added to water to improve the ionization efficiency.

3.3. Method validation

Method validation is one of the basic requirements universally recognized as a necessary part of a comprehensive system to ensure quality and reliability of the results for all analytical applications[1]. The objective of an analytical method validation is to assure that the method is suitable for its intended purpose, such as implementation of legislation and for monitoring and risk assessment studies. It must be ensured that the method generates meaningful data and is accurate, specific, precise, reproducible and robust over the specified range in which an analyte will be analyzed.

3.3.1. System suitability test

The system suitability test (SST) represent an integral part of the method and it is used to ensure adequate performance of chromatographic system. SST is performed during the method validation and also to verify the validation status each time when the analytical procedure is applied. It should check the critical parameters of the method and to ensure the correct performance of the analytical procedure. These criteria should be fulfilled before and/or during the analyses of the samples. In case of LC, usually a standard solution is injected onto chromatographic system in 6 or 8 replicates and the results are further evaluated. Relative standard deviation for replicate injections is evaluated for the variation of retention times and areas. Moreover, efficiency (number of theoretical plates), selectivity, resolution, symmetry factor and repeatability are evaluated during SST. The recommended values for ST parameters are shown in Table 2. In the case when parameters of SST not fulfilling the criteria, the Pharmacopoeia sets which various parameters of chromatographic condition may be modified to satisfy the SST criteria.

SST PARAMETER	**RECOMMENDED VALUE**
Retention time – repeatability RSD (%)	RSD < 1%
Area – repeatability RSD (%)	RSD < 1%
Number of theoretical plates	N > 2000
Symmetry factor	A = 0.8 – 1.5
Resolution	R > 1.5

Table 2: The required values of parameters evaluated during SST.

[1] The sources for this chapter are [93][94][95].

Factors which may affect the chromatographic behavior include the composition of mobile phase, ionic strength, temperature and apparent pH of the mobile phase, flow rate, column length, temperature and pressure, and stationary phase characteristics including porosity, particle size and type of particles.

3.3.2. Method validation

The International Conference on the Harmonisation of Technical Requirements for the registration of Pharmaceuticals in Human Use (ICH) has prepared guidelines for analytical method validation in order to synchronize terms and definitions as well as determination the basic requirements between Europe, the United States of America and Japan. The validation of analytical procedures depends on the type of analytical procedure such as (Table 3) [93]:

TYPE OF ANALYTICAL PROCEDURE	*Minimum number*	***Identification tests***	***Testing for impurities***		***Assay procedures***
VALIDATION CHARACTERISTIC			Quantitative test for impurities content	Limit test for the control of impurities	
Accuracy	9 (e.g.3 x 3)	-	+	-	+
Precision					
- *Repeatability*	6 or 9 (e.g.3 x 3)	-	+	-	+
- *Intermediate precision*		-	+	-	+
Specificity	Not applicable	+	+	+	+
Limit of detection	Approach dependent	-	-[a]	+	-
Limit of quantitation		-	+	-	-
Linearity	5	-	+	-	+
Range	Not applicable	-	+	-	+
Robustness		+	+	+	+

Table 3: List of validation characteristics normally evaluated for the different types of analytical procedures and minimum number of determinations required; (-) - signifies that this characteristic is not normally evaluated, (+) - signifies that this characteristic must be normally evaluated, (a) – may be needed in some cases [93].

Typical validation characteristics tested according to the intended purpose of the method are listed below: accuracy, precision (repeatability, intermediate precision and

reproducibility), specificity, linearity, range, limit of detection (LOD), limit of quantitation (LOQ), robustness and system suitability test.

Revalidation of an analytical method has to be done when changes in the synthesis of the drug substance, changes in the composition of the final drug or changes in the analytical procedure are done. The degree of revalidation depends on the nature of the changes.

Specificity is an ability of a method to differentiate between the analyte and other components which may be expected to be present. Typically these might include impurities, degradation products, matrix, etc. Lack of specificity of an individual method may be compensated by other supporting analytical procedures.

For chromatographic analysis, representative chromatograms of standard solution, blank and sample should be used to demonstrate specificity and selectivity of the method. Available impurities can be spiked at appropriate levels to the corresponding matrix or else degraded samples can be used. The specificity of the method is demonstrated that the result is unaffected by the spiked material and when no peak in blank occurred at the same retention time as analyte and impurities are separated individually and/or from other matrix components and resolution between two peaks which elute closest to each other is higher than 1.5.

The **accuracy** of an analytical procedure expresses the closeness of an agreement between the value measured and the accepted reference value. It should be assessed by comparison of results obtained by an independent procedure or on samples spiked with known amount of analyte. Accuracy should be reported as percentage of recovery between the values measured and accepted true value together with the confidence intervals.

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range of the analytical procedure (e.g. 3 concentrations/3 replicates each of the total analytical procedure).

The **precision** of an analytical procedure expresses the closeness of agreement between series of measurements obtained from multiple sampling of the sample under the prescribed conditions. It is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of series of measurements. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

- **Repeatability** expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision. It should be assessed using a minimum of 9 determinations covering the specified range for

the analytical procedure (e.g. 3 concentrations/3 replicates each) or a minimum of 6 determinations at 100% of the test concentration.

- **Intermediate precision** expresses within-laboratory variations such as different days, different analysts, different equipment, etc. The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used.
- **Reproducibility** expresses the precision between laboratories and should be considered in case of the standardization of analytical methodologies.

The **linearity** of an analytical procedure is its ability (within a given range) to obtain results which are directly proportional to the concentration (amount) of analyte in the sample. The **range** of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure provides an acceptable degree of linearity, accuracy and precision. To determine it, a calibration curve is usually prepared by analyzing a minimum of 5 concentrations of analyte between 50 and 150% of the target analyte concentration. Statistical parameters such as the coefficient of correlation, y-intercept and slope of the regression line should be submitted. For an acceptable linearity range, the correlation coefficient should be greater than 0.9990 and the y-intercept less than 2% of the target concentration response. Moreover, a graphical presentation of the data is recommended.

The **limit of detection (LOD)** is the lowest analyte concentration in a sample which can be detected but not necessarily quantitated as an exact value. The **limit of quantitation (LOQ)** is the lowest amount of analyte in a sample which can be quantitatively determined at an acceptable level of accuracy and precision.

There are several approaches for the determination of LOD and LOQ. The most widely used approach based on signal-to-noise ratio is used for LC. Determination of the signal-to-noise ratio is performed by comparison measured signals from samples with known low concentrations of analyte with those of blank samples. LOD is established as the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio 3:1 is generally considered acceptable for estimating the detection limit. LOQ is established as the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

The **robustness** of an analytical procedure is one of the most important validation parameters. It shows the ability of an analytical method to remain unaffected by small changes in method parameters while it still generates accurate, specific and precise data.

The evaluation of robustness should be considered already during the method development and it depends on the type of procedure under study. In case of LC an influence of variations of pH of mobile phase, buffer concentration, mobile phase composition (percentage of organic modifier), different columns, temperature and flow rate should be tested. Representative chromatograms should be submitted in validation report if there is any important parameter which influences the method performance.

4. RESULTS AND DISCUSSION

This doctoral thesis is mainly focused on the analysis of water samples in order to monitor the presence of pharmaceuticals especially antibiotics in water environment. Completely new methods were developed for the determination of fluoroquinolones and tetracyclines in wastewaters and real samples were analyzed in order to predict a risk assessment from the occurrence of selected antibiotics in the environment.

Another problem is the incomplete elimination of pharmaceuticals and other compounds during wastewater treatment process. For this reason it is necessary to investigate the processes and to improve the treatment process in the future. A study of degradation of fluoroquinolone antibiotic pefloxacin by UV photolysis, ozonation and their combination was done in order to evaluate the efficiency of advanced oxidation processes which could be included into wastewater treatment process.

4.1. An overview of analytical methodologies for the determination of antibiotics in environmental waters

The presence of antibiotics in the environment especially in environmental waters and soils is of increasing concern during last years. Therefore an extensive research about the occurrence, fate and effects of antibiotics in the environment is carried out. For this reason it is necessary to use analytical methods suitable for the monitoring of the antibiotics in the aquatic environment.

The aim of the review was to summarize presently published work about the determination of antibiotics in aquatic environment. The review has been focused on the determination of 4 groups of antibiotics: fluoroquinolones, tetracyclines, macrolides and sulfonamides together with trimethoprim. As the analysis of antibiotics in the environment has usually been carried out by LC-MS a sample preparation step before proper analysis was an essential step. For this reason, the review is divided into two parts: sample preparation and analytical methods.

A short overview of problems of occurrence of antibiotics in the environment is given including sources of antibiotics contamination and their effects in the environment. Sampling and storage of environmental samples is discussed briefly as the review more emphasis the sample preparation step and analytical methods. The first part dealing with the sample preparation has discussed mainly the pH adjustment of samples and further SPE, which is the mostly used technique for the sample preparation as it allows concentration of analytes and clean-up of matrix from interferences. Generally, multiresidue methods are used for analysis of environmental samples allowing the

determination of the emerging antibiotics belonging to the different classes. A total of four tables are included in this first part, where SPE procedures used are summarized. In addition, each group of antibiotics is discussed separately in the point of view of chemical properties which influence the SPE procedure. The second part is dealing with analytical methods for the determination of antibiotics in waters. It includes four tables as well. A short discussion of liquid chromatography and mass spectrometry is given as well as the problems of matrix effects and use of internal standards in environmental analysis. The tables summarize transparently the analytical conditions and each group of antibiotics is again discussed separately.

This review has shown an increasing concern of antibiotic residues determination. LC-MS/MS is the mostly used technique for the analysis of environmental contaminants as it allows sensitive and selective identification and quantitation of antibiotic residues.

This work was published in journal Analytica Chimica Acta (Supplement I) [75].

4.2. Fluoroquinolone antibiotics

4.2.1. Determination of FQs by HPLC-FD

The main goal of this work was to develop a new method for the determination of four fluoroquinolones in wastewater samples using HPLC-FD. Three FQs used in human medicine neither (OFLO, NOR, CIPRO) and veterinary antibiotic ENRO were included in this study. The analysis was done by HPLC-FD as FQs are naturally highly fluorescent compounds. SPE was used during sample preparation step.

The water samples after acidification to pH 4.5 and addition of Na_2EDTA were extracted through an anion-exchange cartridge in tandem with a polymeric Oasis HLB cartridge. Employing an anion-exchange cartridge, where humic acids and other highly negatively charged natural organic matter were retained, prevented contamination, blockage and overloading of Oasis HLB sorbent. The clean-up efficiencies were studied to adjust following parameters such as effect of pH on retention of FQs, the effects of the solvents and pH used in the preconditioning and washing steps and finally the nature and volume of the elution solvent. After the sample percolation, the cartridges were dried and anion-exchange cartridge was removed and washing and elution step was performed only with Oasis HLB cartridge. The separation of four FQs was performed through a monolithic column (Chromolith Performance RP-18e, 100 x 4.6 mm). The FQs were eluted isocratically using a mobile phase composed of 0.025M phosphoric acid solution (pH 3), methanol and acetonitrile (920:70:10) at a flow rate of 1.2 mL/min at room temperature. The fluorimetric detection was performed at an excitation wavelength of 278 nm and an emission wavelength of 450 nm.

The method was completely validated and applied for analysis of samples collected during spring and autumn season from 4 hospitals and influent and effluent from WWTP in Coimbra, Portugal. In all samples residues of CIPRO were found in the high concentrations, which ranged from 127.0 to 10962.5 ng/L. The second most detected antibiotic was NOR (in 79% of samples) in concentrations between 29.6 and 228.9 ng/L, followed by OFLO (in 50% of samples) between 353.3 and 10676.0 ng/L. The high concentration of CIPRO and OFLO detected are in agreement of high usage of those antibiotics in human medicine. ENRO was found at very low concentration, under the LOQ value in hospitals wastewaters, and the high level, 447.0 ng/L, was found in influent from WWTP.

Differences in antibiotic concentrations detected were observed in wastewaters from different hospitals. Hospital 1 and 2 showed higher concentration of antibiotics detected in comparison to Hospital 3. Moreover, a seasonal influence was observed and was evident especially for Hospital 4. The antibiotic concentrations detected were higher

in samples collected during spring season than the antibiotic concentrations in samples collected during early autumn season. The results obtained from hospitals 1 and 2 showed comparable values of CIPRO and NOR with the exception of OFLO. It was observed in higher concentration in sample collected during autumn season. Concerning samples from hospital 3, the OFLO level was higher concentration in autumn in comparison to CIPRO which was detected in higher concentration in sample collected during spring season.

Moreover, the samples from municipal WWTP were analyzed. NOR, CIPRO and ENRO were detected in all samples although in lower concentration levels than in hospital wastewaters samples. This result is explained by a dilution effect of hospital wastewaters with a municipal wastewater or by a degradation process in the aquatic environment. Regarding the seasonal influence, CIPRO and ENRO were detected at lower concentration levels during autumn season. NOR was detected in higher concentration in autumn season than in samples collected during spring. The efficiency of treatment process was evaluated. The reduction of FQs was 85% and 93% for NOR, 54% and 76% for CIPRO and 53% and 56% for ENRO in spring and autumn season, respectively. These results are in agreement with other reported studies. The higher reduction efficiency was observed in early autumn season when the weather was unusually warm.

The results of this study were published in scientific journal Analytical and Bioanalytical Chemistry (Supplement II) [34].

4.2.2. Determination of FQs by UHPLC-MS and UHPLC-FD

The aim of the work was to develop a new method using UHPLC with MS and FD for the determination of five FQs in wastewaters. Initially, systematic method development approach was compared with a conventional one. During the systematic method development, a possibility of automatic switching among four independent analytical columns of different chemistries has been used. In this study reverse-phase C_{18} column BEH C_{18}, BEH Shield RP_{18} with embedded polar phase, phenyl-hexyl analytical column BEH Phenyl, and silica-based analytical column HSS T3 were tested. Moreover two organic modifiers acetonitrile and methanol and two buffers (acidic and basic) were tested in gradient mode of elution. Within 5 hours, 14 different chromatograms including all variables (column, buffer and organic modifier) were generated. Surprisingly the best separation of 5 FQs was obtained on phenyl analytical column at pH 10.5. This is a completely novel approach for the separation of FQs as usually the analysis is performed at acidic pH. The method was further optimized and final separation was performed using ammonium acetate (pH 10.5) and methanol in mobile phase. The separation was performed on BEH Phenyl analytical column (50 x 2.1 mm, 1.7 μm) in gradient mode of elution. As a consequence a new SPE procedure was developed for the sample preparation using basic pH 10.5 as well. The fluorimetric detection was performed at an excitation wavelength of 278 nm and an emission wavelength of 450 nm

The second task of this work was to compare of sensitivity of FD and MS detection. At basic pH, UHPLC-MS/MS was found about two orders of magnitude more sensitive than FD. It is known that FQs demonstrate higher fluorescence in acidic comparing the basic pH. For this reason an assay comparing the sensitivity of FD at acidic and basic pH was done. FD at basic pH 10.5 demonstrated lower sensitivity than at acidic pH, which is conventionally performed.

Both methods, UHPLC-MS/MS and UHPLC-FD were validated and subsequently UHPLC-FD was used for the evaluation of stability of FQs. UHPLC-MS/MS method was used for analysis of wastewater samples.

The results of this study were published in scientific journal Journal of Separation Science (Supplement III) [96].

4.2.3. Occurrence of FQs in hospital wastewaters and WWTP

The aim of this work was to analyze samples from WWTP in Hradec Králové during different seasons to evaluate the occurrence of 5 FQs. The UHPLC-MS/MS method mentioned above was employed in this research [96]. In total fifteen 24-hours composite wastewater samples were analyzed by this method. The samples were collected from influent, effluent and aeration tank from a WWTP of Teaching hospital in Hradec Králové during early summer 2009, autumn 2009 and late winter 2010. The main task of the WWTP is to remove the dangerous property of the wastewater – infectivity. Treated wastewater flows directly to the municipal WWTP for the whole city Hradec Králové. The seasonal effects and the efficiency of WWTP were evaluated.

Concerning the frequency of detection, CIPRO and OFLO were found in all the samples analysed (100%) and NOR in 93% (Figure 28).

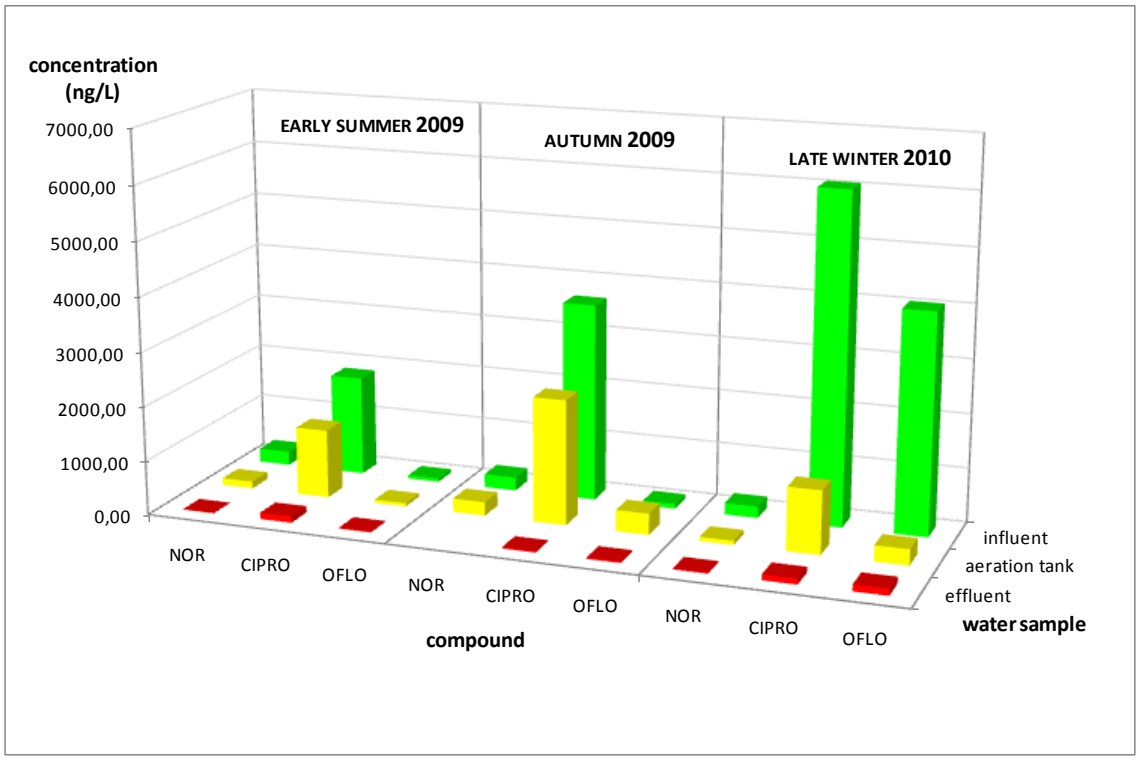


Figure 28: The concentration of FQs detected in samples from wastewater treatment plant.

The highest concentrations were found for CIPRO and OFLO, which is in agreement with their use in human medicine in the Czech Republic. CIPRO was found in the highest levels ranging from 25.1 to 6606.80 ng/L. OFLO was present at high concentrations as well between 15.75 and 4062.69 ng/L and NOR was found in concentrations ranging between 12.40 and 1142.05 ng/L. ENRO and PEFLO were either detected under the LOD or they were not found in the samples. This is in accordance with their use as PEFLO is not very commonly used in the Czech Republic and ENRO is used only for veterinary purposes. A

seasonal influence on the frequency of detection was not observed, all antibiotics were detected in all seasons. However, the detected concentration values showed higher levels during autumn and winter season which is in agreement of their high usage during those seasons.

Concerning the efficiency of treatment process in WWTP the reduction of FQ levels during the treatment process could be evaluated. The efficiency was between 93% and 100% for all compounds (Figure 29). There was no difference of the efficiency of treatment process in dependence on the season. However, lower efficiency of treatment process was observed (70% and 60% for OFLO during early summer and autumn season, respectively).

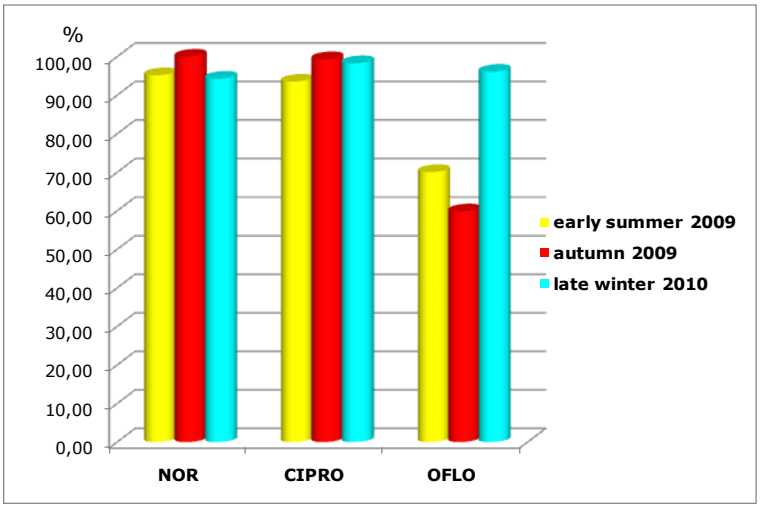


Figure 29: Efficiency of WWTP of Teaching hospital in Hradec Králové.

The samples from aeration tank were analyzed as well. The WWTP is a mechanical-biological sewage treatment plant with complete aerobic sludge stabilization (hydraulic retention time is approximately 8 hours) and accumulation of FQs in the tank could be observed. It is in agreement with other studies as it was suggested that sorption to sewage sludge through hydrophobic interactions is the main removal pathway for FQs. The concentration found in the sample from aeration tank were ranging from 75.96 to 253.45 ng/L for NOR, 1164.27 – 1267.87 ng/L for CIPRO and between 59.26 and 390.95 ng/L for OFLO. Although the accumulation of FQs was observed, very low concentrations of FQs have been found in effluent samples showing the good removal efficiency of WWTP. However, there might be a risk if the sludge from WWTP is dispersed on fields and agricultural areas. It can affect the micro-organisms and can contaminate soil and ground water. Additionally, after rainfall incidents surface waters can be polluted with human or veterinary drugs by run-off from fields treated with digested sludge and those antibiotics can get back into the surface water and consequently to drinking water.

Comparison of fluoroquinolones detected in Portugal and Czech Republic

There were performed two studies monitoring of fluoroquinolone antibiotics in hospital wastewaters and wastewater treatment plant in Coimbra, Portugal and Hradec Králové, Czech Republic. The goal of the studies was to analyze samples of hospital wastewaters and WWTPs collected during different seasons to evaluate seasonal occurrence and efficiency of WWTPs.

A total of 14 wastewaters samples collected during spring and autumn from four hospitals and from influent and effluent water from municipal WWTP in Coimbra were analysed. A total of 15 wastewater samples collected from influent, effluent and aeration tank from a WWTP of Teaching hospital in Hradec Králové during early summer, autumn and late winter were analysed. The results are shown in Table 4.

COMPOUND	COIMBRA (PORTUGAL)		HRADEC KRÁLOVÉ (CZECH REPUBLIC)	
	Frequency of detection (%)	***Concentration detected (ng/L)***	***Frequency of detection (%)***	***Concentration detected (ng/L)***
Ciprofloxacin (CIPRO)	100	100.8 – 10962.5	100	25.1 – 6606.8
Norfloxacin (NOR)	79	29.6 – 455.0	93	12.4 – 1142.1
Ofloxacin (OFLO)	50	353.3 – 10675.5	100	15.8 – 4062.7
Enrofloxacin (ENRO)	29	53.7 – 447.0	-	-

Table 4 : Concentration of fluoroquinolone antibiotics detected in Portugal and Czech Republic.

Concerning of frequency of detection, CIPRO was found in all the samples analysed, followed by NOR and OFLO. ENRO was found only in samples from Portugal. It was not surprising that the highest concentration detected was for CIPRO and OFLO, which are the most used fluoroquinolone antibiotics. As it can be seen from the Table 4, CIPRO and OFLO were found in higher concentration levels than NOR in both studies. ENRO has been found only in samples from Portugal. FQs have been reported in hospital wastewaters in different countries (Table 5). It can be seen that CIPRO and OFLO have been detected in high concentration levels in hospital wastewaters.

COUNTRY	MATRIX	ANTIBIOTIC	LEVELS (ng/L)	REFERENCE
Germany	Hospital wastewater		3000 – 87000	[99]
Sweden	Hospital wastewater	CIPRO	3600 – 101 000	[27]
USA	Hospital wastewater	OFLO	4900 – 34500	[9]
		CIPRO	850 - 2000	

Table 5: Examples of FQs in hospital wastewaters in different countries.

A seasonal influence of the frequency of detection and concentration levels of FQs in hospital wastewaters was observed. It was evident especially for OFLO, NOR and CIPRO in studies from both countries (Table 6, Table 7).

SPRING SEASON				
COMPOUND	HOSPITAL 1	HOSPITAL 2	HOSPITAL 3	HOSPITAL 4
OFLO	2289.0	3008.3	353.3	10675.5
NOR	228.9	-	134.5	29.7
CIPRO	2893.0	15545.5	1926.9	127.0 – 10962.5
ENRO	-	-	-	-
AUTUMN SEASON				
COMPOUND	HOSPITAL 1	HOSPITAL 2	HOSPITAL 3	HOSPITAL 4
OFLO	9451.9	3111.6	1585.5	301.6
NOR	334.0	197.3	88.5	418.8
CIPRO	2927.2	1007.3	619.9	121.8
ENRO	-	-	-	-

Table 6: Concentrations (ng/L) of FQs in water samples collected during spring and autumn season in Portugal (adapted from [34]).

COMPOUND	SPRING	AUTUMN	WINTER
NOR	257.4	249.6	212.4
CIPRO	1851.2	3646.6	6074.4
OFLO	62.3	84.4	4062.7

Table 7: Concentrations (ng/L) of FQs in hospital wastewater samples collected during spring, autumn and winter season in Czech Republic.

Concerning the samples from Portugal, antibiotic levels show comparable levels during spring and autumn. However, higher levels CIPRO and OFLO were detected in some samples collected during spring and autumn, respectively. This might be explained by administration of different antibiotics in different season. Concerning the samples collected in Czech Republic, a seasonal influence was evident for CIPRO and OFLO.

Although NOR was found in similar values in samples from all season. The highest concentration levels of CIPRO and OFLO were found in samples from winter season which is in agreement with high usage of those antibiotics in winter season.

Influent and effluent samples from municipal WWTP in Coimbra, Portugal and hospital WWTP in Hradec Králové, Czech Republic were collected and analyzed. The results are shown in Table 8 and Table 9. Samples collected in Portugal contained FQs in lower levels than in hospital wastewater samples. These results might be explained by a dilution with municipal wastewater, or degradation processes in the aquatic environment.

	SPRING			AUTUMN		
COMPOUND	INFLUENT	EFFLUENT	EFFICIENCY (%)	INFLUENT	EFFLUENT	EFFICIENCY (%)
NOR	191.2	29.6	85	455.0	35.0	92
CIPRO	667.1	309.2	54	418.8	100.8	76
OFLO	-	-	-	-	-	-
ENRO	447.1	211.5	53	121.8	53.7	56

Table 8: Concentrations (ng/L) of FQs in samples from municipal WWTP collected during spring, autumn in Portugal (adapeted from [34]).

	SPRING			AUTUMN			WINTER		
COMPOUND	INF	EFF	EFFICIENCY (%)	INF	EFF	EFFICIENCY (%)	INF	EFF	EFFICIENCY (%)
NOR	257.4	12.4	95	659.8	-	100	320.9	18.3	94
CIPRO	1851.2	119.7	93	4031.4	26.6	99	6340.6	106.9	98
OFLO	62.3	18.7	70	71.0	41.3	60	3116,8	122.3	96
ENRO	-	-	-	-	-	-	-	-	-

Table 9: Concentrations (ng/L) of FQs in samples from hospital WWTP collected during spring, autumn and winter season in Czech Republic (INF = influent, EFF = effluent).

Regarding the seasonal influence, FQs studied were detected at higher levels during the spring season in Portugal and winter season in Czech Republic. These results are equivalent to those mentioned above. Concentrations of FQs detected in municipal WWTP in Portugal are comparable with the results published in scientific literature [34]. The FQs levels in samples from WWTP from Czech Republic are high as the WWTP receives wastewater directly from hospital without any dilution effect.

FQs are eliminated during water treatment process by sorption to sewage sludge through hydrophobic interaction. This statement is supported by the results obtained

from analysis of samples from aeration tank of WWTP in Hradec Králové. These results have been already discussed above.

The reduction of FQs concentrations was observed. The treatment efficiencies from municipal WWTP in Portugal and hospital WWTP in Czech Republic were comparable for NOR and the treatment efficiency for CIPRO was lower in Portugal. The reduction of FQs was 85-95% for NOR, 54-98% for CIPRO, 53-56% for ENRO and 60-96% for OFLO (Table 8 and Table 9). These results correlate well with other reported studies (Table 10). The efficiency of treatment process might vary for the different classes of antibiotics mainly due to differences of chemical structure and properties. However, the elimination rate for FQs is generally in range from 70 to 90%. A lower removal rate of OFLO has been observed in WWTP in Hradec Králové (70 and 60 % during spring and autumn, respectively). This correlates very well with the study from Sweden, where OFLO has been removed (56%) during treatment process [30]. As it has been already said, FQs are known to sorb to sewage sludge which may explain their higher removal rate which is confirmed by several studies [22][30].

COUNTRY	ANTIBIOTIC	REMOVAL EFFICIENCY (%)	REFERENCE
Switzerland	CIPRO	88	[100]
	NOR	92	
Switzerland	CIPRO	70-80	[63]
USA	OFLO	77	[9]
Sweden	NOR	87	[26]
	CIPRO	86	
	ENRO	87	

Table 10: Examples of reduction of FQs levels in WWTP from different countries.

The results of the monitoring of fluoroquinolone antibiotics in Portugal and Czech Republic underscore the importance of this type of monitoring studies. The relatively high antibiotic concentrations have been found in hospital wastewaters supporting the statement that hospitals are primary and very important contributors of antibiotics and other pharmaceuticals into the municipal wastewaters and environment. Moreover, the results of evaluation of treatment efficiency of WWPTs showed the importance to provide more studies in order to evaluate efficiency of the different types of treatment processes and to avoid the entrance of antibiotic residues into the aquatic environment.

4.2.4. Degradation of pefloxacin by advanced oxidation processes

The occurrence of antibiotics in the environment especially in effluent from WWTPs has shown the necessity to improve treatment processes to avoid the entrance of antibiotics into surface waters. Sorption to sludge has been reported to be a major process controlling FQs removal during wastewater treatment [97]. However, sorption is incomplete and FQs have been widely detected in treated wastewater effluents [34][96]. Thus, AOPs as an additional procedure during wastewater treatment can accomplish the water treatment process and avoid the release of FQs into environmental waters.

In this study, degradation of fluoroquinolone antibiotic pefloxacin was investigated. The comparison of ozonation (O_3), UV photolysis at 254 nm (UV) and their combination (O_3/UV) was performed to compare the efficiency of those processes. Moreover the total mineralization (TOC) was studied to get information about the rate of mineralization of PEFLO. In addition the identification of the degradation products was done using MS/MS.

The maximum absorption maxima of PEFLO are by the wavelengths of 272 and 324 nm. The analysis of samples by UV spectrophotometry taken during the degradation experiment has shown the decrease of the absorption intensity of PEFLO while the irradiation time increases (Figure 30). During the decomposition of PEFLO it can be observed a formation of products which have an absorbance at 272 nm as well. For this reason it was necessary to follow the degradation of PEFLO by HPLC-UV. Moreover, limited change in UV spectrum compared to UV spectrum of PEFLO means that chromophore is unaffected and the degradation occurs at the piperazinyl substituent of the oxazinyl group.

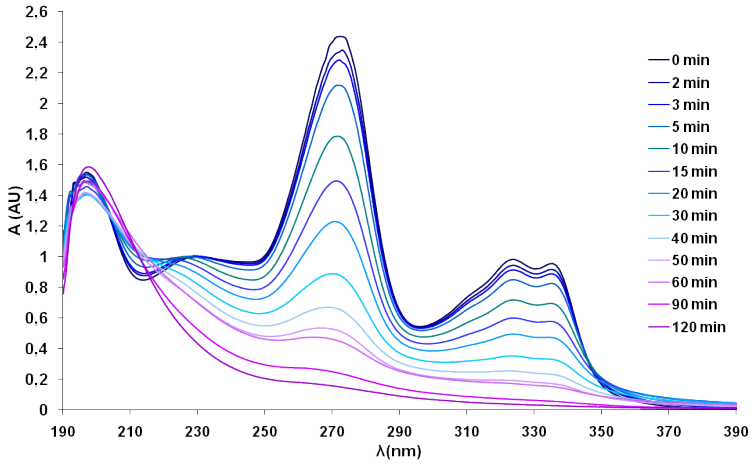


Figure 30: The change of UV spectra during ozonation of PEFLO.

The HPLC analysis used in this study was based on previously developed UHPLC analysis [96]. This method used a mobile phase at basic pH resulting in faster analysis and

better peak shape. In addition, the higher ozone consumption occurs at higher pH. It is due to the higher rate of ozone decomposition into the reactive hydroxyl radicals [74]. The analysis took 20 minutes and the decrease of PEFLO concentration was followed.

The degradation experiments were performed with initial PEFLO concentrations of 0.005, 0.001 or 0.05 mM in AmAc solution pH 9.2 by all three degradation methods (O_3, UV and O_3/UV). The fastest PEFLO degradation can be found at the lowest initial PEFLO concentration (Table 11 and Figure 31).

PEFLO concentration (mol/L)	O_3		UV		O_3/UV	
	$k(s^{-1})$	$t_{1/2}$ (s)	$k(s^{-1})$	$t_{1/2}$ (s)	$k(s^{-1})$	$t_{1/2}$ (s)
$5 \cdot 10^{-5}$	0.002479	200	0.004363	180	0.006369	110
$1 \cdot 10^{-5}$	0.004042	90	0.005499	150	0.009022	70
$5 \cdot 10^{-6}$	0.006450	60	0.005886	140	0.014385	60

Table 11: The degradation constants of PEFLO during O_3, UV and O_3/UV.

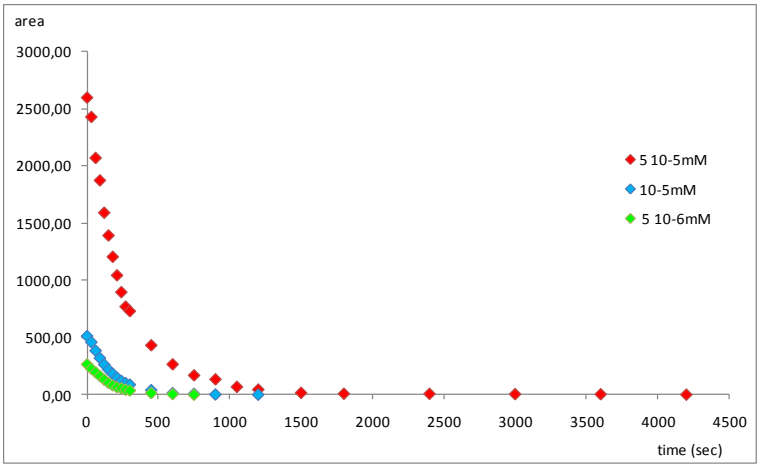


Figure 31: The dependence of efficiency of UV photolysis on PEFLO initial concentration.

The comparison of efficiency of O_3, UV and O_3/UV has shown that O_3 was the most efficient method for the PEFLO degradation, followed by O_3/UV and UV photolysis (Figure 32).

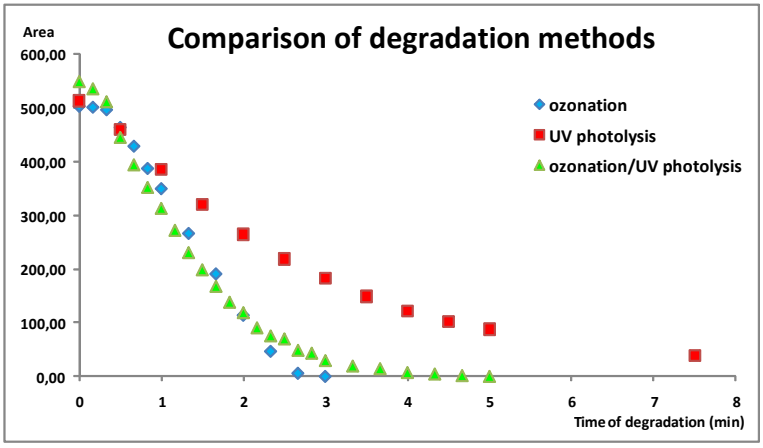


Figure 32: Comparison of degradation methods.

Moreover, the total organic carbon analysis (TOC) has been done to get information about the rate of mineralization of 0.05 mM initial PEFLO concentration for all degradation methods. TOC can be suitable for the comparison of the efficiency of the methods investigated. However, the total degradation of pefloxacin was observed during all three degradation experiments, TOC analysis showed no mineralization at all (Figure 33).

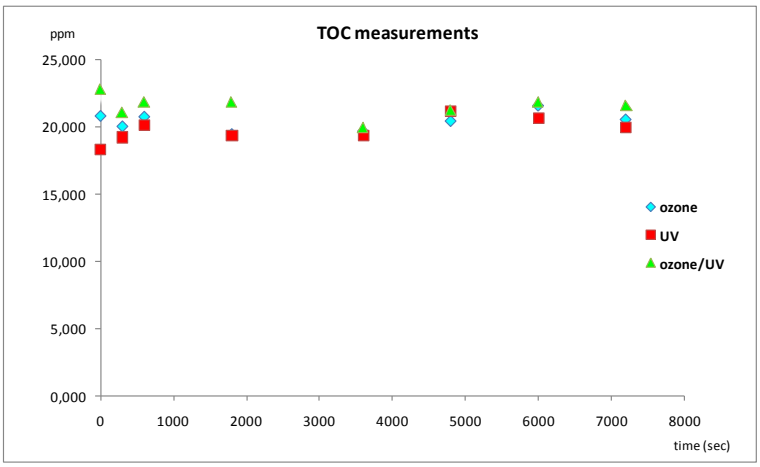


Figure 33: TOC measurement of PEFLO initial concentration 0.05M.

These results might be explained by the transformation of PEFLO into stable organic by-products. For the confirmation of this suggestion, the measurement of presence of aliphatic organic acids was done. The samples taken in the same intervals at those for TOC analysis and were analyzed. Aliphatic carboxyl acid oxalic acid was detected in the 60 min or even 120 min sample of the photolysis and ozonation of PEFLO (c_0 = 0.05 mM) in high amount, followed by maleic, acetic, propionic, formic and fumaric acid (Figure 34).

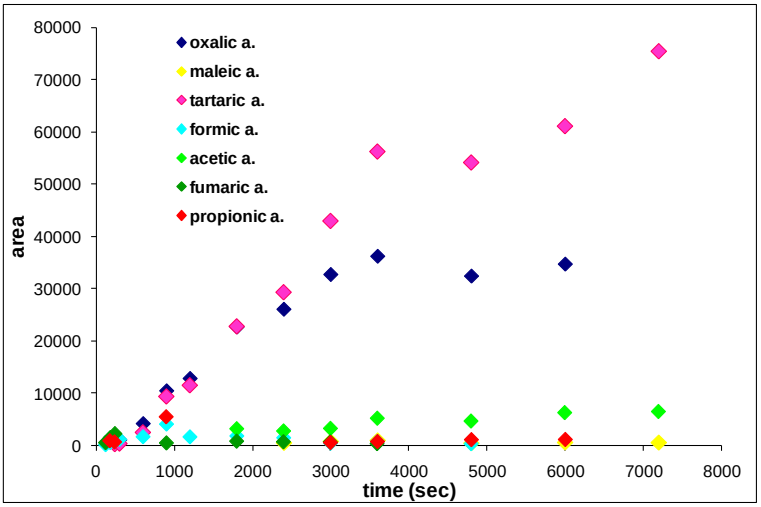


Figure 34: Aliphatic organic acids formed during O_3/UV degradation experiment.

This explained the limited change in the TOC value even the HPLC-UV analysis shown the complete degradation of PEFLO. Although the total mineralization could be suitable during the water treatment process, the formation of hydrophilic degradation products of no toxicity could be also suitable for the treatment process in wastewater treatment plants.

The identification of degradation products was done by UHPLC-MS/MS. Six main degradation products were separated and identified (Figure 35). The most intensive degradation product NOR originates from PEFLO by losing methyl group (Figure 36). Three degradation products of the same mass were identified. These degradation products contain in the structure one more oxygen.

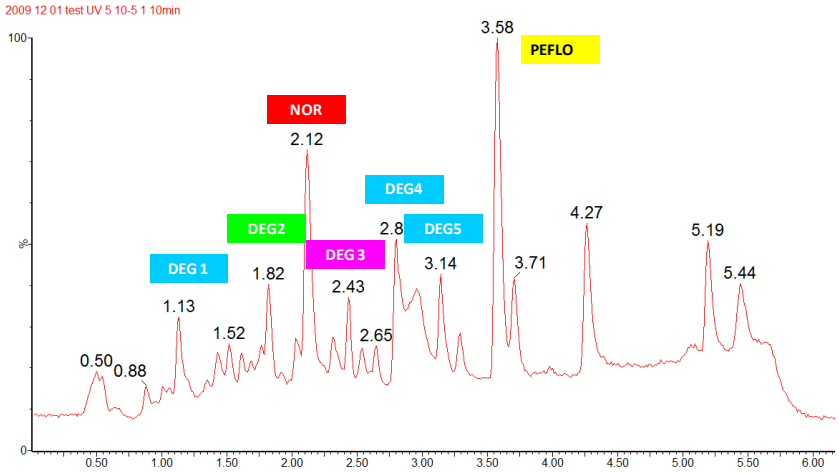


Figure 35: Chromatogram of UHPLC analysis of sample from UV degradation of initial PEFLO concentration 0.05M at 10 minute.

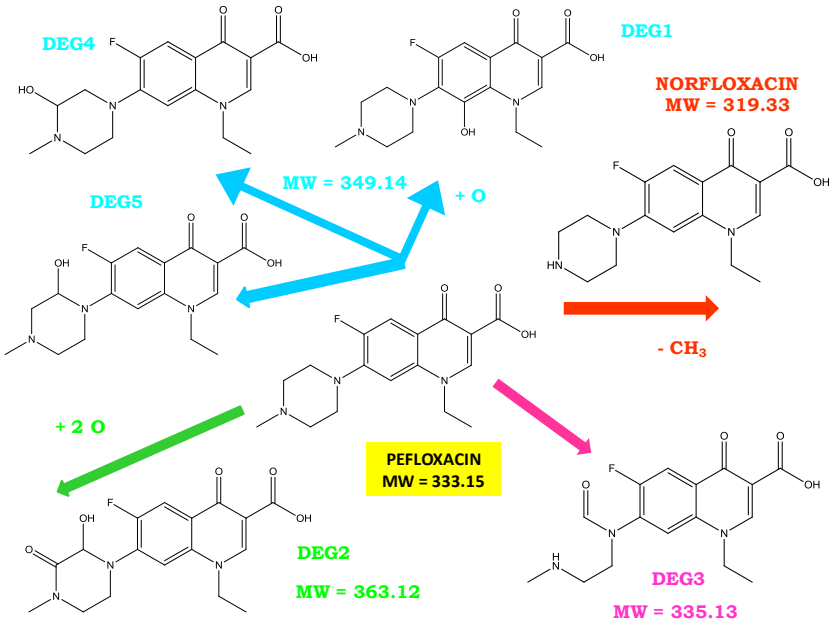


Figure 36: Proposed structures of degradation products of PEFLO.

4.3. Tetracycline antibiotics

4.3.1. Determination of TCs by HPLC-FD

The main goal of this study was to develop a method for the determination of TCs in wastewaters. Analysis was carried out by LC-FD with post-column derivatization. Four TCs were included in this study; tetracycline (TET), its epimer epi-tetracycline (eTET), minocycline (MINO) and doxytetracycline (DOXY).

TCs were separated on a C_8 analytical column by gradient elution with 0.02M oxalic acid and acetonitrile as mobile phase at a flow rate of 1.0 mL/min at room temperature. TCs are naturally weak fluorescent compounds however they show fluorescence due to their ability to form chelate complexes with metal ions, such as Mg^{2+}, Ca^{2+}, Cu^{2+} or Al^{3+} under neutral to slightly alkaline conditions. For this reason a post-column derivatization with magnesium acetate at pH 9.0 (borate buffer) was performed to produce highly fluorescent complexes. The detection was carried out at an excitation wavelength of 386 nm and an emission wavelength of 500 nm.

SPE was used for extraction and clean-up of samples. Wastewater samples after acidification to pH 3.4 and an addition of Na_2EDTA were percolated through polymeric Oasis HLB cartridges. An advantage of Oasis HLB cartridges it that they do not contain silanol groups thus TCs cannot be bound there irreversibly.

The method was completely validated and applied for analysis of samples collected during spring and autumn season from 4 hospitals and influent and effluent from WWTP in Coimbra, Portugal. A total of 24 samples were analyzes and the results are shown in Table 12. Concerning the frequency of detection, MINO and TET were detected in 41.7% of samples, followed by 25% and 8.3% of detection for eTET and DOXY, respectively.

MINO was found in the highest concentrations, which ranged from 95.8 to 915.3 ng/L. TET was found in concentrations between 23.2 and 158.0 ng/L. The detection of epimer or TC (eTET) between 6.0 and 18.9 ng/L indicated the isomerization of TC due to epimer formation. DOXY was found at very low concentration 8.1 ng/L in one hospital wastewater. Regarding the seasonal occurrence, the TCs were detected more frequently and at higher concentration levels in samples collected during spring season. It should be mentioned that during autumn season when samples were collected was unusually warm and dry. This could explain almost no detection of TCs as they are photodegradable very easily when the sunlight is present.

SPRING SEASON						
COMPOUND	**HOSPITAL 1**	**HOSPITAL 2**	**HOSPITAL 3**	**HOSPITAL 4**	**WWTP INFLUENT**	**WWTP EFFLUENT**
MINO	317.79	531.7	-	-	915.3	95.8
eTET	17.5	-	-	6	-	-
TET	158	-	-	29.2	-	-
DOXY	-	-	-	-	-	-
AUTUMN SEASON						
COMPOUND	**HOSPITAL 1**	**HOSPITAL 2**	**HOSPITAL 3**	**HOSPITAL 4**	**WWTP INFLUENT**	**WWTP EFFLUENT**
MINO	-	-	-	-	350	-
eTET	-	18.9	-	-	-	-
TET	42.2	54.7	-	23.2	-	-
DOXY	8.1	-	-	-	-	-

Table 12: Concentrations of TCs (μg/L) residues detected in water samples collected during spring and autumn season in Portugal (adapted from [101]).

Moreover, the samples from municipal WWTP were analyzed. Only MINO was detected and the wastewater treatment resulted in a reduction of 100% and 89.5% during spring and autumn season, respectively.

The results of this study were published in scientific journal Analytical and Bioanalytical Chemistry (supplement IV) [101]. The extended abstract from the XIV. International Symposium on Luminescence Spectrometry was published in journal Luminescence (Supplement V) [102].

4.3.2. Determination of TCs by UHPLC-MS/MS

The goal of this study was to develop a new method for the determination of 6 TCs in wastewaters using UHPLC-MS/MS technique. SPE and UHPLC and MS/MS conditions were studied and optimized. This study involved 6 most important TCs: minocycline (MINO), doxycycline (DOXY), oxytetracycline (OXY), tetracycline (TET), chlortetracycline (CTC) and demeclocycline (DEME).

LC conditions were optimized carefully in the means of optimization of additives, their concentrations, flow rate and gradient profile. The optimal separation of TCs was achieved using C_{18} analytical column (100 mm x 2.1 mm, 1.7 µm) with 0.05% formic acid and ACN as mobile phase in gradient mode of elution at 30°C (Figure 37).

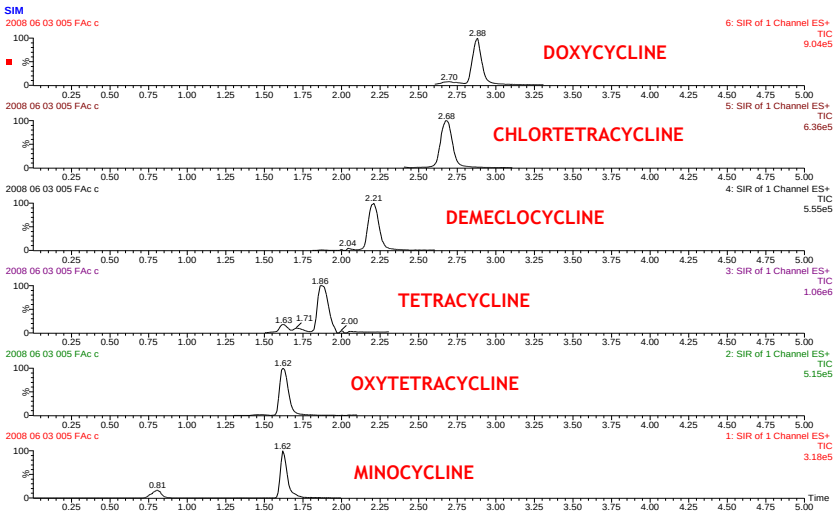


Figure 37: Final separation of 6 TCs by UHPLC-MS/MS.

The detection of TCs was done by MS using ESI in positive mode of ionization. Individual MS parameters were tuned carefully in order to achieve optimal peak intensities and S/N ratios. Final quantitation was done using SRM mode. The spectra of each TC are shown in Figure 38.

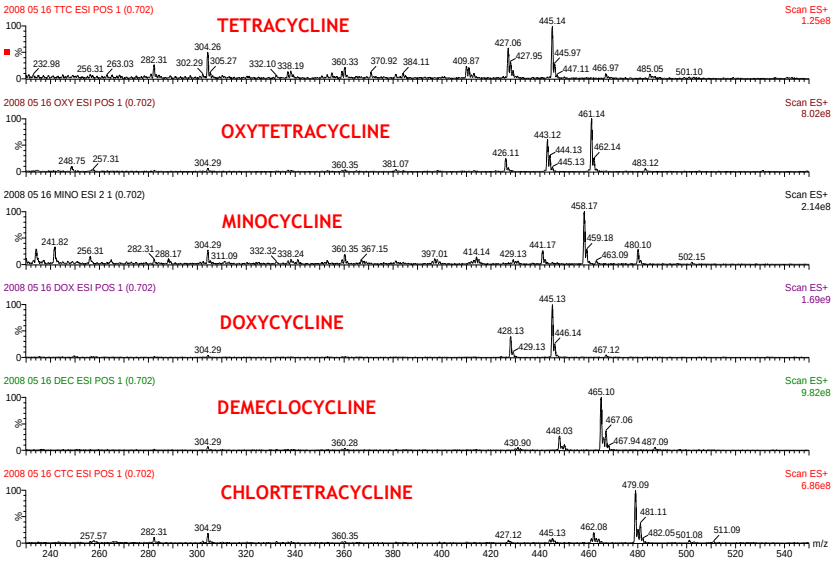


Figure 38: The spectra of selected 6 TCs.

The validation of method was done in terms of sensitivity and repeatability. The limit of detection (LOD) range was 0.1-5 ng/mL and the limit of quantification (LOQ) was 1-25 ng/mL for TCs analyzed.

4.3.3. Determination of TCs by UHPLC-UV and UHPLC-FD

The aim of this part of work was to develop an UHPLC method with fluorescence detection for the determination of 6 TCs in wastewaters. As it was already mentioned above, TCs show fluorescence in complexes with metal ions under neutral or slightly alkaline conditions. For this reason it was necessary to perform a derivatization when TCs were analyzed by LC-FD. There are two possible ways: post-column or in-column derivatization. As TCs show tailing when separation is performed in alkaline conditions a post-column derivatization is preferably used because chromatographic separation occurs at acidic conditions while the reagents to increase the fluorescence are added post-column in an alkaline pH. In addition, an advantage of post-column derivatization is that analytes are better separated from interferences prior to derivatization. However, an in-column derivatization was optimized in order to analyze TCs by UHPLC-FD.

The separation of 6 TCs by UHPLC-UV system was performed at acidic pH. The best separation was achieved using C_8 analytical column with formic acid and methanol as mobile phase (Figure 39). Different analytical columns were compared in point of view separation and sensitivity. BEH Shield RP18 analytical column could be used for the separation of the selected TCs, however, the sensitivity was lower in comparison to the separation on BEH C_8 analytical column (Table 13).

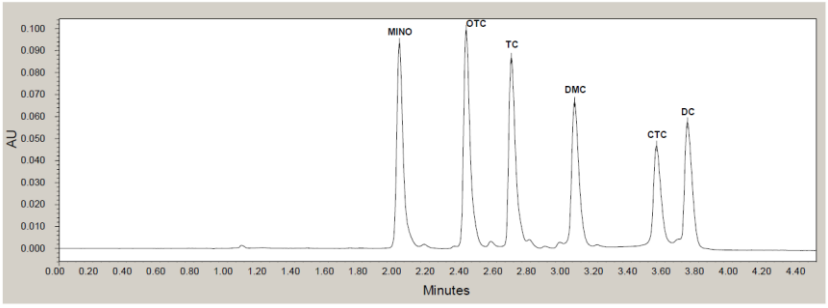


Figure 39: The final separation of 6 TCs by UHPLC-UV at acicid pH using C8 analytical column.

UHPLC-UV (ACIDIC pH AND BEH C_8 COLUMN)						
	MINO	***OXY***	***TET***	***DEME***	***CTC***	***DOXY***
SST						
Resolution	—	6.05	3.95	—	6.15	—
Asymmetry factor	1.50	1.61	1.59	1.28	1.44	—
t_R	2.02	2.42	2.69	3.06	3.55	3.73
Repeatability t_R (% RSD)	0.09	0.06	0.06	0.05	0.05	0.05
Repeatability A (% RSD)	0.12	0.11	0.21	0.17	0.45	0.36
VALIDATION						
Linearity (r^2)	0.9997	0.9998	0.9995	0.9998	0.9996	0.9994
Linearity – range (µg/mL)	0.100-100	0.050-100	0.050-100	0.050-100	0.100-100	0.100-100
LOD (µg/mL)	0.100	0.050	0.050	0.050	0.100	0.100
LOQ (µg/mL)	0.300	0.150	0.150	0.150	0.300	0.300
UHPLC-UV (ACIDIC pH AND BEH SHIELD RP18 COLUMN)						
	MINO	***OXY***	***TET***	***DEME***	***CTC***	***DOXY***
SST						
Resolution	—	8.34	2.44	7.03	6.34	11.0
Asymmetry factor	1.19	1.08	1.15	0.98	1.14	0.90
t_R	1.82	2.48	2.70	3.38	3.95	4.73
Repeatability t_R (% RSD)	0.04	0.03	0.02	0.02	0.02	0.02
Repeatability A (% RSD)	0.11	1.57	1.90	0.19	0.21	0.40
VALIDATION						
Linearity (r^2)	0.9997	0.9997	0.9996	0.9997	0.9995	0.9998
Linearity – range (µg/mL)	0.100-100	0.050-100	0.050-100	0.100-100	0.100-100	0.100-100
LOD (µg/mL)	0.100	0.050	0.050	0.100	0.100	0.100
LOQ (µg/mL)	0.300	0.150	0.150	0.300	0.300	0.300

Table 13 : The validation parameters and system suitability test of UHPLC-UV analysis of TCs on C18 and Shield RP18 analytical column.

During the optimization of UHPLC-FD separation the complexation with Ca^{2+} and Mg^{2+} cations was tested. As it was impossible to perform post-column derivatization, the in-line derivatization was examined. The separation of 7 FQs was achieved by using C_8 analytical column (100 mm x 2.1 mm, 1.7 µm). During the method optimization different parameters were optimized including pH of ammonium acetate, concentration of Ca^{2+} and EDTA. The final mobile phase contained a pH 7 ammonium acetate buffer with 35mM $CaCl_2$ and 25 mM EDTA (solvent A) and methanol. The separation was performed in gradient mode of elution and FQs were eluted in 5 minutes (Figure 40).

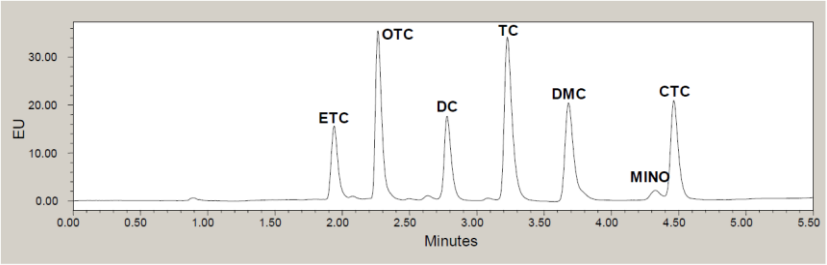


Figure 40: The separation of 7 TCs by UHPLC-FD on C8 analytical column.

System suitability test was done for both methods and parameters such as resolution, symmetry factor, peak capacity, repeatability of retention times and areas were evaluated. During the validation, linearity was evaluated and LOD and LOQ established. Table 13 and Table 14 summarize the results obtained during the validation of UHPLC-UV and UHPLC-FD methods, respectively.

	UHPLC-FD (COMPLEXATION WITH CALCIUM AND EDTA, PH 7.5 AND BEH C_8 COLUMN)					
	OXY	***DOXY***	***TET***	***DEME***	***MINO***	***CTC***
SST						
Resolution	—	5.09	3.95	2.80	3.91	3.13
Asymmetry factor	1.48	1.67	1.45	1.43	1.45	1.23
t_R	2.18	2.66	3.06	3.36	3.82	4.20
Repeatability t_R (% RSD)	0.06	0.12	0.26	0.24	0.19	0.06
Repeatability A (% RSD)	0.50	0.55	0.65	0.67	0.57	0.96
VALIDATION						
Linearity (r^2)	0.9999	0.9996	0.9992	0.9996	0.9993	0.9996
Linearity – range (µg/mL)	0.050-100	0.050-100	0.050-100	0.050-100	1-200	0.075-100
LOD (µg/mL)	0.0167	0.0167	0.0167	0.0167	1	0.025
LOQ (µg/mL)	0.050	0.050	0.050	0.050	3	0.075

Table 14 : The validation parameters and SST of UHPLC-FD analysis of 6TCs.

5. APPENDIX

5.1. List of papers included in this doctoral thesis

1. Supplement I (page 98)

Marcela Seifrtová, Lucie Nováková, Celeste Lino, Angelina Pena, Petr Solich

An overview of analytical methodologies for the determination of antibiotics in environmental waters

Anal. Chim. Acta 649 (2009) 158-179.

(IF: 3.757)

Times cited: 5

2. Supplement II (page 121)

Marcela Seifrtová, Angelina Pena, Celeste Lino, Petr Solich

Determination of fluoroquinolone antibiotics in hospital and municipal wastewaters in Coimbra by liquid chromatography with a monolithic column and fluorescence detection

Anal. Bioanal. Chem. 391 (2008) 799-805.

(IF: 3.480)

Times cited: 28

3. Supplement III (page 129)

Marcela Seifrtová, Jana Aufartová, Jitka Vytlačilová, Angelina Pena, Petr Solich, Lucie Nováková

Determination of fluoroquinolone antibiotics in wastewater using ultra high-performance liquid chromatography with mass spectrometry and fluorescence detection

J. Sep. Sci. 33 (2010) 2094-2108.

(IF: 2.551)

Times cited: 1

4. Supplement IV (page 145)

Angelina Pena, Miguel Paulo, Liliana Silva, Marcela Seifrtová, Celeste Lino, Petr Solich

Tetracycline antibiotics in hospital and municipal wastewaters: a pilot study in Portugal

Anal. Bioanal. Chem. 396 (2010) 2929-2936.

(IF: 3.480)

Times cited: 2

5. Supplement V (page 154)

Angelina Pena, Liliana Silva, Marcela Seifrtová, Celeste Lino, Petr Solich

Determination of tetracycline antibiotics in hospital and municipal wastewaters in Coimbra, Portugal by liquid chromatography with fluorescence detection: occurrence and seasonal influence

Luminescence 25 (2010) 237-238.

(IF: 1.209)

5.2. Publications not included in this doctoral thesis

1. Angelina Pena, Marcela Seifrtová, Celeste Lino, Irene Silveira, Petr Solich
Estimation of Ochratoxin A in Portuguese Population: New Data on the Occurrence in Human Urine by High Performance Liquid Chromatography with Fluorescence Detection
Food Chem. Toxicol. 44 (2006) 1449-1454.

(IF: 2.114)

Times cited: 23

2. Lucie Nováková, Zdeněk Spáčil, Marcela Seifrtová, Lubomír Opletal, Petr Solich
Rapid qualitative and quantitative ultra high performance liquid chromatography method for simultaneous analysis of twenty nine common phenolic compounds of various structures
Talanta 80 (2010) 1970-1979.

(IF: 3.290)

Times cited: 2

5.3. Oral presentations

1. M. Seifrtová
   Occurrence of antibiotics residues in hospital and municipal waste waters from Coimbra
   „Seminários de Pós-Graduação 2007“ University of Coimbra, Faculty of Pharmacy, Coimbra, Portugal, 27.06.2007

2. M. Seifrtová, A. Dombi, K. Gajda-Schrantz, E. Illés, L. Nováková, P. Solich
   Degradation of fluoroquinolone antibiotic pefloxacin by UV photolysis, ozonation and their combination
   36th International Symposium on Environmental Analytical Chemistry, 05.-09.10.2010, Rome, Italy

5.4. Poster presentations at international scientific conferences

5.4.1. Fluoroquinolone antibiotics

1. M. Seifrtová, A. Pena, S.M. Lino, P. Solich
   Determination of fluoroquinolone antibiotics in hospital and municipal wastewaters in Coimbra by liquid chromatography with fluorescence detection using monolithic column
   IX International Symposium on Analytical Methodology in the Environmental Field, 03.-05.10.2007, Pollensa, Mallorca, Spain

2. M. Seifrtová, A. Pena, C. Lino, P. Solich
   Occurrence of fluoroquinolone antibiotics in hospital and municipal wastewaters in Coimbra, Portugal
   AOAC Europe Workshop: Enforcement on European Legislation on Food and Water: Analytical and Toxicological Aspects, 17.-18.04.2008, Lisbon, Portugal

3. A. Pena, J. Aufartová, M. Seifrtová, C. Lino, P. Solich
   Determination of fluoroquinolone antibiotics in different types of soil samples
   19th International Symposium on Pharmaceutical and Biomedical Analysis, 08.-12.06.2008, Gdańsk, Poland

4. L. Nováková, A. Pena, M. Seifrtová, P. Solich
   Optimization of methodology for the determination of four fluoroquinolone antibiotics in wastewater by UPLC-MS/MS
   35th International Symposium on Environmental Analytical Chemistry, 22.-26.06.2008, Gdańsk, Poland

5. M. Seifrtová, L. Nováková, A. Pena, P. Solich
   UPLC-MS/MS method for the determination of fluoroquinolones in environmental waters
   International ESTROM Conference, 03.-05.09.2008, Bucharest, Romania

6. J. Vytlačilová, M. Dubánková, M. Seifrtová
   Determination of fluoroquinolone antibiotics in wastewater of Hradec Králové hospital and their ecotoxicology testing
   SETAC Europe, 19th Annual Meeting, 31.05.-04.06.2009, Göteborg, Sweden

7. M. Seifrtová, J. Aufartová, L. Nováková, A. Pena, P. Solich
   UPLC automatical method development versus conventional aproach – optimization and development of analytical methodology for the determination of fluoroquinolone antibiotics
   12th EuCheMS International Conference of Chemistry and the Environment, 14.-17.06.2009, Stockholm, Sweeden

8. J. Aufartová, M. Seifrtová, L. Nováková, P. Solich
   Comparison of HPLC and UPLC method for the determination of fluoroquinolones. Automatic method development and classical approach
   HPLC 2009, 28.06.-02.07.2009, Dresden, Germany

9. M. Seifrtová, J. Aufartová, A. Pena, L. Nováková, P. Solich
   Development of new UHPLC-MS/MS method for the determination of antibiotics in environmental waters
   25th International Symposium of Microscale Bioseparations MSB 2010, 21.-25.03.2010, Prague, Czech Republic

10. L. Nováková, M. Seifrtová, H. Vlčková, J. Aufartová, P. Solich
    Systematic method development is crucial in modern pharmaceutical analysis
    70th FIP World Congress of Pharmacy/ Pharmaceutical Sciences, 28.08.-02.09.2010, Lisbon, Portugal

5.4.2. Tetracycline antibiotics

11. A. Pena, M. Paulo, M. Seifrtová, C.M. Lino, P. Solich
    Determination of minocycline, tetracycline, epitetracycline and doxycycline antibiotics in hospital and municipal wastewaters in Coimbra by liquid chromatography with fluorescence detection
    IX International Symposium on Analytical Methodology in the Environmental Field, 03.-05.10.2007, Pollensa, Mallorca, Spain

12. A. Pena, R. Albert-Garcia, M. Seifrtová, C. Lino, P. Solich, M. Calatayud
    Comparison of two analytical methods for determination of TCs antibiotics in environmental wastewaters
    19th International Symposium on Pharmaceutical and Biomedical Analysis, 08.-12.06.2008, Gdańsk, Poland

13. H. Andrade, L. Nováková, M. Seifrtová, P. Solich, C. Montenegro
    Development of a new methodology for the determination of six tetracycline antibiotics in hospital wastewaters using-MS/MS
    IJUP 09, 2nd Meeting of Young Researches at UP, 25.-27.02.2009, Porto, Portugal

14. M. Seifrtová, H. Andrade, L. Nováková, P. Solich
    UPLC-MS/MS method for the determination of tetracycline antibiotics in environmental waters
    12th EuCheMS International Conference of Chemistry and the Environment, 14.-17.06.2009, Stockholm, Sweden

15. M. Seifrtová, A. Pena, L. Silva, C. Lino, P. Solich.
    Tetracycline antibiotics in hospital and municipal wastewaters in Coimbra, Portugal: Occurrence and seasonal influence
    XIV. International Symposium on Luminiscence Spectrometry, 13.-16.07.2010, Prague, Czech Republic

5.5. Supplement I

Marcela Seifrtová, Lucie Nováková, Celeste Lino, Angelina Pena, Petr Solich

An overview of analytical methodologies for the determination of antibiotics in environmental waters.

Anal. Chim. Acta 649 (2009) 158-179.

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Review

An overview of analytical methodologies for the determination of antibiotics in environmental waters

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ABSTRACT

The widespread occurrence of antibiotics as contaminants in the aquatic environment has increased attention in the last years. The concern over the release of antibiotics into the environment is related primarily to the potential for the development of antimicrobial resistance among microorganisms. This article presents an overview of analytical methodologies for the determination of quinolone (Qs) and fluoroquinolone (FQs), macrolide (MLs), tetracycline (TCs), sulfonamide (SAs) antibiotics and trimethoprim (TMP) in different environmental waters. The analysis of these antibiotics has usually been carried out by high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) and to a lesser extent by ultraviolet (UV) or fluorescence detection (FD). A very important step before LC analysis is sample preparation and extraction leading to elimination of interferences and prevention of matrix effect and preconcentration of target analytes.

Contents

Abbreviations: AcAc, acetic acid; ACN, acetonitrile; AmAc, ammonium acetate; APCI, atmospheric pressure chemical ionization; AZI, azithromycin; CF, concentration factor; CIN, cinoxacin; CIPRO, ciprofloxacin; CLAR, clarithromycin; CLIN, clindamycin; CTC, chlortetracycline; CZE, capillary zone electrophoresis; DAD, diode-array detector; DAN, danofloxacin; DEME, demeclocycline; DIF, difloxacin; DOXY, doxycycline; ENO, enoxacin; ENRO, enrofloxacin; ERY, erythromycin; ESI, electrospray ionization; FAc, formic acid; FD, fluorescence detection; FLE, fleroxacin; FLU, flumequine; FQs, fluoroquinolone antibiotics; GAT, gatifloxacin; HPLC, high-performance liquid chromatography; IS, internal standard; LC, liquid chromatography; LEV, levofloxacin; LLE, liquid–liquid extraction; LOD, limit of detection; LOME, lomefloxacin; LOQ, limit of quantification; MAR, marbofloxacin; MCX, mixed-cation exchange; MECLO, meclocycline; MeOH, methanol; MINO, minocycline; MLs, macrolide antibiotics; MOXI, moxifloxacin; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NAL, nalidixic acid; NOR, norfloxacin; OFLO, ofloxacin; OXO, oxolinic acid; OXY, oxytetracycline; PEFLO, pefloxacin; PIP, pipemidic acid; Qs, quinolone antibiotics; RIA, radioimmunoassay; ROXI, roxithromycin; SAD, sulfanilamide; SARA, sarafloxacin; SAs, sulfonamide antibiotics; SAX, strong-anion exchange; SCP, sulfachloropyridazine; SCT, sulfacetamide; SDM, sulfadimidine; SDT, sulfadimethoxine; SDX, sulfadoxine; SDZ, sulfadiazine; SGN, sulfaguanidine; SIM, selected ion monitoring; SM, sulfameter; SMD, sulfamethoxydiazine; SML, sulfamoxole; SMM, sulfamonomethoxine; SMO, sulfamethoxine; SMP, sulfamethoxypyrazine; SMR, sulfamerazine; SMT, sulfamethizole; SMX, sulfamethoxazole; SMZ, sulfamethazine; SNT, sulfanitran; SPAR, sparfloxacin; SPE, solid-phase extraction; SPIR, spiramycin; SPME, solid-phase microextraction; SPY, sulfapyridine; SQX, sulfaquinoxaline; SRM, selected reaction monitoring; SSM, sulfisomidin; SSX, sulfisoxazole; SSZ, sulfasalazine; STZ, sulfathiazole; TCs, tetracycline antibiotics; TET, tetracycline; TLS, tylosin; TMP, trimethoprim; TOS, tosufloxacin; UHPLC, ultra-high-performance liquid chromatography; UV, ultraviolet; VAN, vancomycin; WCX, weak-cation exchange; WWTPs, wastewater treatment plants.

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1. Introduction

Pharmaceuticals represent a group of emerging chemicals of environmental concern widely used in human and veterinary medicine. They can enter the environment either as parent compounds or metabolites, conjugates or both. Pharmaceuticals have been found in surface waters and wastewaters at levels of up to a few $\mu g\,L^{-1}$. Antibiotics, followed by steroid compounds, analgesics/nonsteroidal and anti-inflammatory drugs, are the most widely studied classes of pharmaceuticals [1].

Antibiotics are an important group of pharmaceuticals in today's medicine and have been detected in various compartments of the aquatic environment, e.g. wastewaters, surface and ground water and in drinking water as well [2–4]. They are regarded as "pseudopersistent" contaminants due to their continual input into the ecosystem. Therefore, the occurrence of antibiotics in the environment has received considerable attention. They are generally poorly absorbed by the human body and thus excreted either unchanged or transformed, via urine and faeces [5]. There is a growing interest about their presence, persistence and fate in the environment because low levels of antibiotics can favor the proliferation of antibiotic resistant bacteria. The use of antibiotics in animal agriculture has been linked to the increased emergence of resistant strains of pathogenic bacteria that have potential to impact human health. Resistance genes and/or antibiotic resistant bacteria can be transferred from animals to humans. In addition, bacteria can develop cross-resistance between antibiotics used in veterinary medicine with those of similar structures used exclusively in human medicine [6].

Antibiotics are released to the aquatic environment in different pathways. After the administration to humans, they are excreted as metabolites but also a considerable amount is eliminated in unchanged form as parent compounds via urine and faeces into the sewage. Many researches have shown the incomplete removal of pharmaceuticals during wastewater treatment processes. Wastewater treatment plants (WWTPs) are considered to be major contributors of presence of pharmaceuticals in the environment. Pharmaceuticals along with their metabolites have been found in the effluents from WWTPs [7–9]. Therefore they can reach the surface and groundwater. There is a potential risk for the aquatic and soil organisms which is associated with the presence of trace concentrations of these bioactive compounds. Hospitals are also one of the most important contributors of the occurrence of the antibiotics into the aquatic environment [10,11].

Use of antibiotics in veterinary medicine for the treatment of bacterial infections of animals as well as prophylactic agents is another source of contamination. The animal excreta are the major source of contamination, as the most of these substances end up in manure. The manure and slurry (urine and faeces) are either stored or immediately applied to the agricultural fields as fertilizers. The unmetabolized compounds present in the manure or their biologically active metabolites may move from the manure from the field to the groundwater and eventually enter surface water, such as rivers and lakes and thus they can affect the aquatic organisms. This is depending on their mobility in the soil system. The sludge from WWTPs can be used to fertilize soils as well. In addition, antibiotics are extensively used in fish farms. They are used as feed additives or they are directly applied into the water. The result of an overfeeding is that many compounds end up in the sediments where they are slowly degraded or slowly leach out back into the surrounding waters.

Drugs may persist in solid environmental matrices for a long time. The persistence depends on their photostability, binding and adsorption capacity, degradation rate and leaching into the water. Strongly sorbing pharmaceuticals tend to accumulate in soils or sediment (TCs, FQs) and by contrast, highly mobile pharmaceuticals (SAs) have a potential to resist degradation and tend to leach into the groundwater and to be transported with the groundwater, drainage water and surface water run-off to surface waters [12]. The sorptive exchange of chemicals between a water phase and a solid phase is represented by the sorption coefficient $K_{d,solid}$, which is defined as the ratio between the concentration of the compound in the sorbent and in the water at the equilibrium [13]. Another sorption mechanism is forming complexes between antibiotic and metal ions such as Ca^{2+}, Mg^{2+}, Fe^{3+} or Al^{3+}. This is important for TCs, FQs and for MLs and their persistence in the environment.

The analysis of antibiotics in the environment represents a difficult task due both to the high complexity of the matrices analyzed and to the usually low concentrations ($ng\,L^{-1}$) at which target compounds are present in the environmental waters. This reason makes the development of very sensitive analytical methods suitable for the monitoring of these analytes in low $ng\,L^{-1}$ concentration level necessary. The typical low concentrations of antibiotics found in the environment make a preconcentration step prior to the detection imperative and essential. Off-line solid phase extraction (SPE) is the method of choice for the sample preconcentration and usually it is followed by liquid chromatography (LC) analysis. Nowadays, a new trend became the injection of water samples directly onto HPLC or ultra-high-performance liquid chromatography (UHPLC) system with MS detection. It is possible mainly due to the high sensitivity of MS detection which allows that analytes do not need to be pre-concentrated and can be quantified accurately in water samples.

Many antibiotics from different classes have been found in the aquatic environment. Therefore currently multiresidual analytical methods are preferred for the determination and monitoring of different groups of antibiotics. These methods must be sensitive, selective, not so much time-consuming and easily applicable

to analyze environmental samples. There have already been published reviews dealing with the determination of specific groups of pharmaceuticals in different scientific journals [14,15]. In this review we attempt to summarize recently published analytical methodologies for the extraction and following determination of selected antibiotics in environmental water samples. Antibiotics are characterized according to their structural and chemical properties. Members of the same group have similar structures, act by similar mechanisms, and are likely to behave similarly in the environment. For the purposes of this article following groups of antibiotics were selected: Qs and FQs, TCs, MLs, SAs and TMP. The reason was their widespread usage in the medicine and their occurrence in the aquatic environment. FQs, the second-generation of Qs are synthetic antibiotics. MLs are produced by various *Streptomyces* strains. TCs are broad-spectrum antibiotics highly effective against numerous gram-positive and gram-negative bacteria. Those groups of antibiotics are widely used in human medicine and in veterinary medicine as well. SAs are antibacterial agents, commonly used in veterinary prophylaxis of infections and also in the treatment of diseases. TMP is a dihydrofolate reductase inhibitor structurally different from SAs. It is commonly prescribed in combination with sulfamethoxazole (SMX) (as co-trimoxazole, which contains SMX:TMP in a 5:1 ratio) or it can be prescribed on its own. The β-lactam class of antimicrobials, including penicillins and cephalosporins, are used for the treatment of both humans and animals. However, due to the chemically unstable β-lactam ring, members of the lactam class of antimicrobials readily undergo hydrolysis. These compounds are not commonly detected in environmental waters that is the reason why this review does not deal with them.

2. Sampling, storage and stability

The whole analytical procedure typically includes five steps: sampling, sample preparation, chromatographic separation, detection and data analysis. The most important parts of the analytical process are sampling and sample preparation because they take more than 80% of the analytical time. Sampling is a selection of a small fraction of matrix enough in volume to still accurately represent the part of the environment. Sampling is so important that it can cause the main contribution to the error of the whole analytical process. The main difficulties in the sampling are representativeness and integrity. Possible errors during the sampling step can be caused by choosing improper sampling method, location and frequency of sampling and number of samples collected. Other errors could be originating from storage and handling with the sample. The sampling frequency is an important factor of the representativeness. The low sampling frequency could underestimate the occasional presence of samples with high analyte concentration. Usually, 24-h composite samples are collected in the environmental area. The composite samples are used to exclude a possibility of non-representative results, whereby samples were collected at low tide, dilution would be at a minimum and so not representative sample would be obtained [16].

The preservation of the sample is an additional problem in the sampling process. Several problems which can occur during the sampling and storage step are decomposition by means of temperature, UV irradiation, microbial activity and chemical reactions. The following approaches are applied to preserve the sample integrity. Samples should be protected from the external agents (they are collected in brown amber glass bottles) and stored at low temperature (approx. 4 °C or frozen at −20 °C) in a dark ambient until the chromatographic analysis. These precautions are very important especially for TCs which can be easily degradaded [11]. Storage at higher temperatures can enhance the bacterial growth and activity resulting in a loss of analytes. The other possible approach is an addition of preservatives which should be suitable to avoid decomposition by means of chemical reactions and microbial activity. The samples can be acidified to inhibit a bacterial growth [16]. Hydrochloric acid was used for acidification of the samples up to pH 2.0 prior to the analysis of MLs in the water samples [17]. Sulphuric acid was employed for the pH adjustment of the sample to pH 2.0 for the determination of erythromycin (ERY), SMX and TMP [18]. One method referred the addition of $Na_2S_2O_3$ as a quenching agent to consume residual chlorine contained in the samples of wastewater effluents [19].

A further important factor in the sampling process is a filtration. Generally it is performed when sample arrives to the laboratory. The filtration is usually carried out on 0.45 or 0.2 μm glass-fibre filters. The second filtration could be performed immediately before the sample preparation step or the sample could be centrifuged. The filtration step is necessary to remove particles from the water samples which can plug up the SPE cartridges and thus slow down significantly the sample preparation step. However, the filtration may lead to loss of analytes in case that they are hydrophobic and adsorb to particles in the water samples.

Considering the stability of analytes during the storage, the stability of Qs (oxolinic acid and ciprofloxacin) was determined in river water [20]. The stability has been studied both in containers and on C_{18} SPE cartridges under different storage conditions: time (up to 4 months), light (sunlight *vs.* dark), and temperature (ambient, 4, and −18 °C). SPE cartridges were chosen for the study, because their easier transport and their use would considerably reduce space requirements for storage in laboratories, especially if large-volume samples must be stored at low temperatures. Results showed high influence of temperature and time of storage on the stability of studied compounds. Antibiotics were stable both in the containers and on SPE cartridges for at least 2 weeks at ambient temperature. Stability was increased substantially if samples were stored at low temperatures (4 and −18 °C) and in dark. However stability of antibiotics in SPE cartridges was significantly lower and antibiotics were degraded more quickly when stored in SPE cartridges, than in aqueous samples. After 3 months, significant degradation of analytes was observed even when cartridges were stored at −18 °C comparing to storage in containers, where the concentration of analytes was almost the same after 4 months, both at 4 and −18 °C. These results confirmed the suitability of SPE cartridges for use in the field of sampling of the antibiotics from river water and their subsequent storage, although for a shorter time.

Regarding the stability of standards, the standard solutions should be stored in amber bottles to avoid light penetration, usually below the temperature 4 °C, and warmed to room temperature before use [21]. In some papers the stability study for standards was provided. It is recommended to use the standard solutions not longer than 3 months. For example, TCs can decompose rapidly under the influence of light and atmospheric oxygen, forming degradation products. The stock standard solutions are mostly prepared in methanol (MeOH) and stored at 4 °C for 1 month.

3. Sample pre-treatment and extraction procedure

Sample preparation is the crucial step in environmental analysis. It is highly influenced by the physical and chemical properties of analytes studied and by matrices. The main goal is to concentrate analytes in sample, to remove interferences from matrix and to prepare analyte in suitable form for subsequent chromatographic analysis. Usually, the sample preparation step includes adjustment of solution pH, addition of chelator followed by extraction procedure, handling with the extract and final preparation for following chromatographic analysis. An overview of sample preparation procedures are given in Tables 1–4.

Table 1
Extraction procedures utilized for the sample preparation in multiresidue studies.

Substances isolated	Matrix	Sample pretreatment	Sorbent type	Conditioning solvents	Washing solvent	Elution solvent	Recovery (%), concentration factor	Final analysis	Ref.
CIPRO, DOXY, SMX, TMP, ERY (+28 pharmaceuticals)	Surface water (1 L)	pH adjustment 2.5 (by HCl), addition EDTA	Oasis MCX	2 mL MeOH, 2 mL 2% FAc/water	2 mL 2% FAc/water	1 mL MeOH, 2 mL 5% ammonia/MeOH	Rec = 61.6–82.5%, CF = 2000	UHPLC–MS/MS	[1]
SMX, ERY (+13 pharmaceuticals)	Surface, drinking, ground water (100 mL)	pH adjustment 3.0 (by HCl)	Oasis MCX	5 mL acetone, 5 mL water	6 mL water (pH 3.0)	8 mL MeOH + ammonia (95:5)	Rec = 63–96%, CF = 200	LC–MS/MS	[2]
CIPRO, NOR, ENRO, SARA, PIP, OXO, FLU, MINO, OXY, TET, DEME, CTC, DOXY, STZ, SMR, SMZ, SMT, SCP, SMX, SDT, TMP, ERY, ROXI, TLS	Drinking water (500 mL)	pH adjustment 3.0, addition EDTA	Oasis HLB	6 mL MeOH, 3 mL MeOH + 0.1% FAc, 2× 6 mL water	2× 6 mL water	4× 2 mL MeOH + 0.1% FAc	Rec = 91–161%, CF = 2000	LC–MS/MS	[4]
SDZ, STZ, SMZ, SPY, SMX, N^4-acetyl-SMX, TMP, AZI, CLAR, ERY, ROXI	WWTP: 1st effluent (50 mL), 2nd, 3rd effluent (250 mL)	Dilution with 150 mL of water, addition NaCl, pH adjustment 4.0 (by H_2SO_4)	Oasis HLB	2× 1.5 mL MeOH-EtAc (1:1), 2× 1.5 mL MeOH + 1% ammonia, 2× 1.5 mL water (pH 4.0)	1.5 mL water-MeOH (95:5)	2× 1.5 mL MeOH-EtAc (1:1), 2× 1.5 MeOH + 1% ammonia	Rec = 30–124%, CF = 100, 500	LC–MS/MS	[7]
ERY, CLAR, ROXI (+9 hormones)	WWTP influents and effluents (1 L)		DVB-phobic speedisk cartridges, clean-up = SEC	15 mL MeOH, 15 mL water	15 mL water	15 mL *tert*-butyl methyl ether, 15 mL MeOH	Rec = 81–92%, CF = 1000	LC–MS/MS	[8]
CIPRO, NOR, OFLO, DOXY, SMX, TMP (+β-lactams)	Hospital wastewater (200–500 mL)	pH adjustment 3.0 (by H_2SO_4)	C_2/ENV+	5 mL MeOH, 5 mL MeOH–water (50:50), 5 mL water (pH 3.0)	5 mL water (pH 3.0)	5 mL triethylamine (5%) in MeOH	Rec = 55–87%	LC–MS/MS	[10]
ENRO, OXY, SMZ, SDZ, SCN, TMP (+β-lactams)	Wastewater (100 mL)	pH adjustment 4.0	Oasis HLB	5 mL MeOH, 5 mL water (pH 4.0)	2 mL 2% MeOH	2× 5 mL MeOH	Rec = 89.3–97.9%, Rec_{SCN} = 11.2%, CF = 100	LC–DAD	[12]
SMX, TMP, ERY-H_2O (+27 pharmaceuticals)	Surface water (1 L)	pH adjustment 2.0 (by H_2SO_4)	Oasis HLB	5 mL methyl *tert*-butyl ether, 5 mL water	5 mL water	5 mL MeOH/methyl *tert*-butyl ether (10:90), 5 mL MeOH	Rec = 71–91%, CF = 1000	LC–MS/MS	[18]
CIPRO, ENRO, NOR, OFLO, SMZ, SMX, TMP	WWTP effluents (2nd, 3rd) (1 L)	Addition NaCl, pH adjustment 2.5 (by H_3PO_4)	Anion-exchange cartridge (on the top), Oasis HLB	6 mL MeOH, 6 mL 4.38 mM H_3PO_4		10 mL 95% MeOH/5% 4.38 mM H_3PO_4	Rec = 37–129%, CF = 1000	LC–MS	[19]
OXY, CTC, MINO, DEME, MECLO, TET, DOXY, STZ, SMX, SMR, SCP, SMZ, SDT, ERY, ROXI, TLS	River water (120 mL)		Oasis HLB	3 mL MeOH, 3 mL water	9 mL water	5 mL MeOH	Rec = 76.6–124.8%, CF = 1000	LC–MS/MS	[21]
CIPRO, ENRO, NOR, OFLO, OXO, PIP, CTC, DOXY, OXY, TET, 16 SAs, CLAR, ERY, ROXI	WWTP final effluent (1 L)	pH adjustment 3.0 (by H_2SO_4) (Qs, TCs, SAs), addition EDTA, pH adjustment 6.0 (by H_2SO_4) (MLs)	Oasis HLB	6 mL acetone, 6 mL MeOH, 6 mL 50 mM EDTA (pH 3.0), 6 mL acetone, 6 mL MeOH, 6 mL water (pH 6.0) (MLs)		3× 2 mL MeOH	Rec = 72–99%, CF = 1000	LC–MS/MS	[22]
CIPRO, CTC, TET, SDT, SMZ, SMT, SMX, STZ, SSX, CLAR (+20 pharmaceuticals)	WWTP influent (500 mL) and effluent (1 L)	pH adjustment 6.0 (FQ, MLs), pH adjustment 3.0 (TCs, SAs) (by H_2SO_4)	Oasis HLB	6 mL acetone, 6 mL MeOH, 6 mL water (pH 6) (FQs, MLs), 6 mL 50 mM EDTA (TCs, SAs)		3× 2 mL MeOH	Rec = 57–94%, CF = 500, 1000	LC–MS/MS	[23]

Table 1 (*Continued*)

Substances isolated	Matrix	Sample pretreatment	Sorbent type	Conditioning solvents	Washing solvent	Elution solvent	Recovery (%), concentration factor	Final analysis	Ref.
OFLO, NOR, CIPRO (+5 pharmaceuticals)	Groundwater (1 L), surface water (500 mL), WWTP influent (100 mL) and effluent (250 mL)	pH adjustment 10.0 (by NaOH)	Oasis HLB	2 mL *n*-hexane, 2 mL acetone, 10 mL MeOH, 10 mL non-contaminated groundwater (pH 10.0)	2 mL 5% MeOH in 2% NH_4OH	4× 1 mL MeOH	Rec = 32–97%, CF = 200, 500, 1000, 2000	LC–MS/MS	[24]
CTC, DOXY, MECLO, OXY, TET, SCP, SDT, SMR, SMZ, STZ, TMP, ERY, ROXI, TLS (+pharmaceuticals)	Surface water (400 mL)	Addition EDTA, pH adjustment 8.2 (by H_2SO_4 or NaOH)	Oasis HLB	5 mL MeOH, 5 mL water	5 mL 5% MeOH	5 mL MeOH	Rec = 65–134%, CF = 800	LC–MS/MS	[25]
SMX, TMP, ERY (+13 pharmaceuticals)	Surface water, wastewater	pH adjustment 6.0 (by HCl or aq. ammonia)	Strata X	5 mL MeOH, 5 mL water		5 mL acetone, 2× 5 mL MeOH		LC–MS/MS	[26]
TMP, ERY (+16 pharmaceuticals)	Hospital wastewater (100 mL)	pH adjustment 7.0 (by H_2SO_4)	Oasis HLB	6 mL MeOH, 5 mL water	5 mL water	2× 4 mL MeOH	Rec = 87.9–95.2%, CF = 100	LC–MS/MS	[27]
SMX, TMP (+22 pharmaceuticals)	Surface water, ground water (1 L)		Oasis HLB	6 mL MeOH, 6 mL water	1 mL 5% MeOH/water	3 mL MeOH, 2 mL MeOH (pH 3.7)	CF = 1000	LC–MS	[28]
SPY, SMX, SSX, SMZ, SDT, TMP, ERY, CLAR, ROXI	WWTP influent (100 mL) and effluent (500 mL)		Oasis HLB	3× 2 mL MeOH, 3× 2 mL water		6 mL water	CF = 100, 500	LC–MS/MS	[29]
OXY, CTC, TET, DEME, DOXY, MECL, MINO STZ, SMR, SMZ, SCP, SMX, SDT	Surface waters (120 mL)	Addition EDTA, pH adjustment <3.0 (by H_2SO_4)	Oasis HLB	3 mL MeOH, 3 mL HCl, 3 mL water	3 mL water	5 mL MeOH	Rec = 82.1–101.6%, CF = 1000	LC–MS/MS	[32]
CTC, DEME, DOXY, MECLO, OXY, TET, STZ, SMZ, SCP, SMY, SDT	WWTP influent and effluent (120 mL)	Addition EDTA, citric acid, pH adjustment <3.0 (by H_2SO_4)	Oasis HLB	3 mL MeOH, 3 mL HCl, 3 mL water	3 mL water	5 mL MeOH	Rec_{INF} = 77.9–99.8%, Rec_{EFF} = 83.6–103.5%, CF = 1000	LC–MS/MS	[33]
Acetyl-SMX, SMX, TMP, ERY	Surface water, sewage effluent (1 L)	pH adjustment 3.0 (by HCl)	Strata X	3× 2 mL MeOH, 3× 2 ml water, 3× 2 ml water (pH 3)		3× 2 mL MeOH	Rec = 56–123%	LC–MS/MS	[38,40]
SMX, TMP (+20 pharmaceuticals)	WWTP influent and effluent (500 mL)	pH adjustment 4.0 (by H_2SO_4)	Strata X	6 mL MeOH, 6 mL water	5 mL water	10 mL MeOH	CF = 1000	LC–MS/MS	[39]
OFLO, SMX, TMP, ERY, AZI (+27 pharmaceuticals)	Ground waters (500 mL), WWTP influents (100 mL) and effluents (200 mL)		Oasis HLB	5 mL MeOH, 5 mL water	5 mL water	2× 4 mL MeOH	Rec = 30–116%, CF = 500, 100, 200	LC–MS/MS	[41]
CIPRO, OFLO, CTC, DOXY, OXY, TET, SDM, SMX, TMP, AZI, CLAR, CLIN, ERY, ROXI, SPIR, TLS, VAN	Surface water (500 mL)	pH adjustment 4.0 (by H_2SO_4), addition EDTA	Oasis HLB (on the top), SDB-2	1× cart. MeOH, 3× cart. water	2× catr. water (pH 4.0)	4× 1 mL MeOH, 4× 1 mL MeOH–FAc	Rec = 62–106%	LC–MS/MS	[42]
CIPRO, ENRO, NOR, SARA, CTC, DOXY, OXY, TET, SMT, STZ, SMR, SMZ, SCP, SMX, SDT TMP, ERY-H_2O, ROXI, TLS	Surface water, WWTP influent and effluent (500 mL)	Addition EDTA, pH adjustment 3.0 (by H_2SO_4)	Oasis HLB, MCX	2 mL water, 2 mL MeOH, 2 mL MeOH + 5% NH_4OH, 2 mL reagent water, 2 mL water, pH 3 (H_2SO_4)	2 mL water	6 mL MeOH, +MCX: 2 mL 5% NH_4OH–MeOH	Rec = 71–138%, CF = 10 000	LC–MS	[43]
CIPRO, ENRO, TET, OXY, CTC, SMZ, SDT, SMX, TMP, CLIN, ERY, ROXI, TLS	Surface, ground waters, WWTP effluent (500 mL)	addition EDTA, pH adjustment 2.8–3.0 (by H_3PO_4)	Oasis HLB, tC_{18} Sep-Pak cartridges	6 mL ACN, 6 mL water		4 mL ACN	Rec = 71–117%, CF = 500	LC–MS/MS	[44]
NOR, DOXY, TMP, ERY-H_2O (+β-lactams)	Surface seawater (500 mL)	Addition EDTA, pH adjustment 3.0 (by FAc)	Oasis HLB	4 mL ACN, 4 mL water	4 mL water	4 mL ACN	Rec = 99–116%, CF = 250	LC–MS/MS	[45]

OXO, SDZ, TMP	Surface water (40 mL)	pH adjustment 5.0–5.2 (by H_3PO_4)	Oasis HLB	2 mL MeOH, 1 mL water	2 mL water	3 mL ACN	Rec = 78–84%, CF = 80	LC–MS/MS	[46]
CIPRO, ENRO, CTC, DOXY, OXY, TET	River water (1 L), WWTP influent (100 mL), WWTP effluent (250 mL)	pH adjustment 2.8 (by HCl)	Oasis HLB	5 mL MeOH, 2 mL water		5 mL MeOH	Rec = 88–112%	LC–MS	[47]
CIPRO, NOR, OFLO, DOXY, SMX, TMP, ERY-H_2O (+β-lactams)	WWTP effluent (1 L)	pH adjustment 3.0 (by H_2SO_4)	ENV+	5 mL MeOH, 5 mL 50% MeOH/water, 5 mL water (pH 3.0)	5 mL water (pH 3.0)	2 mL MeOH, 5 mL 5% TEA/MeOH	Rec = 54–101%, CF = 1000	LC–MS/MS	[59]
OFLO, SMX, TMP, AZI, ERY (+29 pharmaceuticals)	River water (500 mL), WWTP influent (100 mL) and effluent (200 mL)		Oasis HLB	5 mL MeOH, 5 mL water	5 mL water	2× 4 mL MeOH	CF = 100, 200, 500	UHPLC–MS/MS	[61]
CIPRO, NOR, LOME, LEV, GAT, SPAR, MOXI, SMX, TMP (+β-lactams)	Surface water (100 mL)	pH adjustment 3.0 (by FAc)	Oasis HLB	5 mL MeOH, 5 mL water (pH 3)		10 mL MeOH	Rec = 86–103%, CF = 200	LC–MS/MS	[66]
ROXI (+novobicin, atorvastatin)	Surface water, WWTP effluent (500 mL)	pH adjustment 4.0 (by H_2SO_4)	Oasis HLB	6 mL acetone, 6 mL MeOH, 6 mL water (pH 4.0)	10 mL water (pH 4.0)	3× 2 mL MeOH	Rec_{SW} = 93%, Rec_{WWTP} = 89%	LC–MS/MS	[67]
TLS (+3 veterinary antibiotics)	Surface water (30 mL)		Oasis HLB	6 mL MeOH, 6 mL water	4 mL water	5 mL MeOH + 2% FAc	Rec = 87–121%	LC–MS/MS	[68]

The pH of sample solution significantly influences the chemical form of analytes in samples, their stability and the interaction between the analyte and SPE cartridge packing material. Therefore, for the preparation of environmental water samples the knowledge of pK_a values of analytes is the most important. Antibiotics from group of FQs, TCs, SAs and MLs have acidic and/or basic functional groups and therefore their ionization is controlled by solution pH. Most antibiotics are acidic substances; thus the acidification 2 units under pK_a values of target analytes in water samples in order to obtain their neutral or acidic forms is required and allows the retention of these substances in the most commonly used SPE sorbent polymeric Oasis HLB columns, whereas the negatively charged organic matter usually present in natural samples can be retained in anionic exchange materials, which improves further retention of target compounds. In majority of multiresidue studies, the sample pH was adjusted in pH range 2.5–4 by sulphuric or hydrochloric acid. Some authors used sample of pH 6.0 or higher usually when MLs were included in the study together with other antibiotics or pharmaceuticals [22–27]. In some studies the best SPE recoveries were reported with no sample pH adjustment [28]. This could be somewhat strange, because each sample has different pH and thus the charge of the analytes differs. McClure and Wong [29] used Oasis HLB cartridges for the extraction of SAs, MLs and TMP from the wastewater samples without pH adjustment and supported that Oasis HLB cartridges were effective at neutral pH for the collection of those antibiotics from environmental waters. However, pH of wastewater samples could be even higher or lower than neutral pH. Thus the recovery could be different.

The following step during the sample pre-treatment is an addition of chelating agent. Environmental matrices contain many compounds including divalent or polyvalent cations. The antibiotics from the group of TCs, FQs and MLs form complexes with those ions. Therefore special precautions have to be taken. Above mentioned antibiotics have been found to be sorbed to the residual metals on SPE cartridges and glassware, resulting in irreversible binding to the cartridge and lowering recovery. To obtain sufficient recovery from the environmental matrices some chelator should be added [30,31]. Chelating agents such as EDTA, oxalic acid and citric acid are usually applied to decrease the tendency for antibiotics to bind to cations in the matrix, to improve peak shape and to prevent interferences during the extraction of antibiotics [22]. The addition of strong chelator EDTA to the sample prior to extraction is mostly utilized to chelate metals or multivalent cations (residual metal ions) that are sufficiently soluble in water. They may be present either in solution or sorbed on the surface of the sorbent. Another way of removing metals is washing them out of the cartridge using a solution of 0.5 M HCl during the precondition step [32,33].

One study refers a salt addition step during the determination of FQs, SAs and TMP in wastewaters [19]. In this case, an addition of 0.1 M NaCl improved antibiotic extraction efficiency, particularly for SAs and TMP. Although the amount of added salt was not sufficient to salt out the antibiotics, the presence of additional electrolytes appeared to facilitated sorption of the antibiotics to Oasis HLB cartridges.

In the most instances, the preconcentration and clean-up has been performed by SPE. SPE has been the mostly preferred technique, which replaced classical liquid-liquid extraction (LLE) and become the most common sample preparation technique in the environmental area. SPE offers some advantages over LLE such as improved selectivity, specificity and reproducibility, lower organic solvent consumption, shorter sample preparation time, and easier operation and the possibility of automation. Solid-phase microextraction (SPME) has been used in some cases [29,34,35].

From the point of view concentration of analytes, the achieved concentration factor is an important parameter. The mostly referred preconcentration factor of sample was 500 or 1000 (see Table 1).

Table 2
Extraction procedures utilized for the sample preparation during FQs analysis.

Substances isolated	Matrix	Sample pretreatment	Sorbent type	Conditioning solvents	Washing solvent	Elution solvent	Recovery (%), concentration factor	Final analysis	Ref.
CIPRO, DAN, ENO, ENRO, NOR, CINO, FLU, NAL, OXO	Surface water (250 mL)	pH adjustment 4.0	C_{18} disk cartridges	2 × 1 mL MeOH, 1 mL water, 1 ml 2 mM sodium acetate buffer (pH 4.0)	3 × 500 μL acetate buffer (pH 4) + 15% ACN	3 × 1 mL 6% ammonia/MeOH	Rec = 87–101%, CF = 1000	LC–UV	[36]
CIPRO, DAN, DIF, ENRO, FLU, MAR, NAL, NOR, OXO, SARA	Surface water (250–500 mL)	pH adjustment 5.5	Oasis HLB	5 mL MeOH, 10 mL water, 5 mL AcAc/acetate buffer (pH 5.5)	10 mL water	2 mL 0.01 M NaOH-ACN (75:25)	Rec = 70–99%, CF = up to 250	LC–FD	[50]
CIPRO, DIF, ENRO, LOME, NOR, OFLO, PIP, SARA, TOS	WWTP effluents, Surface water (150–500 mL)	pH adjustment 3.0 (by FAc)	MCP (mixed-phase cation exchange) cartridges	8 mL MeOH, 8 mL water (pH 3.0)		4 mL 5% ammonia/MeOH	Rec = 75–107%, CF = up to 250	LC–MS, LC–FD	[51]
OFLO, NOR, CIPRO	WWTP effluents (250 mL)	pH adjustment 3.0 (by HCl)	Oasis WCX	4 mL MeOH, 10 mL water (pH 3)	100 mL water (pH 3), 5 mL MeOH	10 mL MeOH/ACN/FAc (20/75/5)	Rec = 87–94%, CF = 250	LC–FD, LC–MS/MS	[52]
OFLO, NOR, CIPRO, ENRO	Wastewater	pH adjustment 4.5 (by H_2SO_4) addition EDTA	SAX, Oasis HLB	2 mL MeOH, 2 mL citric acid (pH 4.0)	2 mL citric acid (pH 4.0), 20 ml water (pH 4.2)	4 mL MeOH	Rec = 96–114%	LC–FD	[53]
NOR, CIPRO, ENRO	Surface water	pH adjustment (by H_2SO_4) addition EDTA	Oasis HLB	5 mL MeOH, 4 mL water	Water (pH 4.0)	4 mL MeOH	Rec = 76.5–97.2%	LC–FD	[54]
CIPRO, ENRO, FLE, FLU, LOME, MOXI, NOR, OFLO, OXO	Surface water, Municipal wastewater, WWTP effluent (500 mL)	Addition EDTA, pH adjustment 4.2 (by AcAc)	Chromabond tetracycline	EtAc, MeOH, 0.2% EDTA (pH 4.2)	5 mL water + 0.2% EDTA (pH 4.2)	2 mL MeOH-water (75:25), 2 mL MeOH	Rec = 81.9–104.9%, CF = 1000	LC–FD, LC–MS	[55]

Table 3
Extraction procedures utilized for the sample preparation during SAs analysis included TMP.

Substances isolated	Matrix	Sample pretreatment	Sorbent type	Conditioning solvents	Washing solvent	Elution solvent	Recovery (%), concentration factor	Final analysis	Ref.
SMT, SMX, SMO, SPY, SDZ, SCP, SMR, SSM, SQX, SMP, SDT	Surface water (250 mL), drinking water (1 L)		Oasis HLB	6 mL MeOH, 6 mL water	1 mL 5% MeOH	10 mL MeOH + 50 mM FAc	Rec = 87–99%, CF = 1000, 4000	LC–MS/MS	[3]
SMZ, SDT, SCP, SMO	Ground water (500 mL)		Oasis HLB	3 mL ACN, 3 mL water		2× 3 mL ACN	Rec = 51–113%, CF = 500	LC–MS/MS	[6]
SGN, SCT, SDZ, SPY, SMR, SMZ, SDT, SSZ	Wastewaters (500 mL)	pH adjustment 3.0 (by H_2SO_4)	MCX sorbent phase	5 mL water, 5 mL MeOH, 5 mL MeOH/5% NaOH, 5 mL water (pH 3.0)	4 mL HCl, 5 mL MeOH	2 mL MeOH/5% ammonia	Rec = 37.3–131%, CF = 2000	LC–MS/MS	[34]
SCT, SDZ, SMX, STZ, SMR, SSX, SMT, SMZ, SMM, SMP, SCP, SDX, SDT, SM	Wastewater (500 mL)		Oasis HLB	3 mL MeOH, 3 mL water	3 mL water	2× 3 mL MeOH	Rec = 22.3–87.0%, CF = 500	LC–MS/MS	[37]
SDZ, SCP, SMT, SQX, SDM, SMD	Surface water (250–500 mL)	pH adjustment 3.4 (by FAc buffer)	Oasis HLB	5 mL MeOH, 5 mL FAc buffer pH 3.5	10 mL water	1 mL ACN	Rec = 73–107%	LC–FD	[57]
SMP, SMO, SQX, SNT, SSM, SMX, SMR, STZ, SDZ, SMT, SDM, SDT, SPY, SSX, SCP, SM, TMP	WWTP influent (250 mL) and effluent (500 mL), River water (1 L)	Addition EDTA	Double SPE, Oasis HLB, Sep-Pak silica	6 mL dichloromethane, 6 mL MeOH, 12 mL 50 mM EDTA, 4 mL hexane	10 mL water, 3 mL hexane, 6 ml hexane/EtAc	6 mL dichloromethane/MeOH (2:1), 3 mL MeOH/acetone (1:1), 3 mL acetone	Rec = 62–102%, CF = 500, 1000, 2000	LC–MS/MS	[58]
SAD, SDZ, STZ, SMR, SMZ, SMP, SDT, SQX	Swine wastewater (150 mL)		Oasis HLB	3 mL MeOH, 3 mL 0.5 M HCl, 3 mL water	1 mL water	5 mL ammonia/MeOH (1:19)	Rec = 31.9–106%, CF = 300	LC–UV	[64]

Table 4
Extraction procedures utilized for the sample preparation during MLs analysis.

Substances isolated	Matrix	Sample pretreatment	Sorbent type	Conditioning solvents	Washing solvent	Elution solvent	Recovery (%), concentration factor	Final analysis	Ref.
CLAR, ROXI, ERY-H_2O, SPIR, TLS	Ground water, WWTP effluents (1 L)	pH adjustment 7.0 (by H_2SO_4 or NaOH)	LiChrolute EN, LiChrolute RP-18	3× 2 mL *n*-hexane, 3× 2 mL MeOH, 6× 2 mL water (pH 3.0)		5× 1 mL MeOH	Rec_{GW} = 59–97%, Rec_{WWTP} = 66–81%	LC–MS, LC–MS/MS	[5]
ERY-H_2O, TLS	WWTP influent (100 mL) and effluent (200 mL)	Addition EDTA, citric acid (pH 6.0), pH adjustment ~5.0 (by H_2SO_4)	Oasis HLB	3 mL MeOH, 3 mL HCl, 3 mL water	5 mL water	5 mL MeOH	Rec = 87–101%, CF = 400, 1000	LC–MS/MS	[9]
AZI, CLAR, ERY, ROXI, JOS	Surface water (250 mL)	pH adjustment 6.0 (by NaOH)	Oasis HLB	5 mL ACN, 5 mL water	10 mL water	1 mL 10 mM AmAc (pH 6.0) + ACN (50:50)	Rec_{AZI} = 65–75%, Rec_{MLs} = 84–115%, CF = 250	LC–MS, LC–MS/MS	[17]
ERY-H_2O, ROXI, TLS	Surface waters, WWTPs (120 mL)	Addition EDTA, pH adjustment 5.0 (by H_2SO_4)	Oasis HLB	3 mL MeOH, 3 mL water	3 mL water	5 mL MeOH	Rec_{SW} = 92–94%, Rec_{WWTP} = 83–86%, CF = 1000	LC–MS/MS	[30]

According to the sample volume used, usually 500 or 1000 mL of surface or ground water. When the wastewater sample was analyzed, lower volumes can be used for preconcentration because antibiotics occur there at higher concentration levels. For example, in the evaluation of efficiency of treatment process in WWTP, only 50 or 100 mL of influent and 250 mL of effluents from WWTPs were used [7,24,29,33]. However, nowadays, the trend is using smaller amount of sample allowing fast sample preparation step and thus shorter time of analysis or even the direct injection of water sample on LC. However, there can be a problem with matrix interferences as wastewater samples are complex matrices.

In spite of this statement, SPE procedure is excellent for the extraction of analytes from aqueous matrix, their preconcentration and clean-up. Clean-up depends on the complexity of the sample matrix. It is known that the matrix compounds present in real samples may affect the interaction of analytes with the sorbent used in SPE processes. Matrix constituents may form complexes with the target compounds, preventing their interaction with the sorbent or, more frequently, matrix components (usually present at high concentration levels) interact with the sorbent reducing the number of free sites available for the retention of the analytes [36]. Another problem encountered in the extraction of antibiotics from wastewater is the matrix interference due to the high amount of organic matter in the samples. Organic matter reduces extraction efficiency and interferes with the detection [18]. The minimal matrix effects for SAs in wastewaters have been shown in one study [33].

Processing by SPE allows simultaneous extraction of multiple samples and generally gives good recovery of target compounds. In the scientific literature there are many multiresidual analytical methods describing the simultaneous analysis of antibiotics from different groups or also with the other pharmaceuticals (Table 1). The greatest difficulty in the multiresidue analysis concerns the choice of the best SPE adsorbent and it is obvious that the optimization of SPE conditions must lead to a compromise because the compounds are characterized by different physicochemical properties. The selection of experimental conditions in some cases does not yield to obtaining the best performance and recoveries for each compound. However, there are several disadvantages associated with SPE of pharmaceuticals from the environmental samples. SPE can be laborious and time-consuming, given large samples volumes (100–1000 mL per sample) and co-extraction of unwanted matrix components which are typically present at much higher concentration than the analytes of interest in matrices such as wastewater.

The selection of the most suitable SPE sorbent has to be done with the respect to the polarity of analytes and the sample matrix. Classical SPE sorbent chemistries range from the chemically bonded silica with the C_8 or C_{18} organic group and ion-exchange materials to the polymeric materials. Silica based sorbents have several disadvantages compared to polymeric sorbents [37]. They are unstable in a broader pH range and contain free silanol groups, which are not suitable for the extraction of TCs because of their irreversible binding to the free silanol groups.

Among various types, Oasis HLB cartridges show the most robust recovery ratio and reproducibility for both polar and non-polar compounds and they are employed in the extraction due to their chemical composition containing the lipophilic divinylbenzene units and the hydrophilic *N*-vinylpyrrolidone units allowing working in wide range of pH (from pH 1 to 14). They do not contain free silanol groups to which many amphoteric pharmaceuticals can be strongly bound and thus cannot be eluted with the conventional organic solvents. They were used in majority of studies (see Table 1) and have been selected for the extraction of TCs and MLs since they are silanol free avoiding the antibiotics binding. Only two works used Oasis MCX (mixed cation exchange) cartridges [1,2]. In four works Strata X cartridges were employed for the extraction of SAs, MLs and pharmaceuticals from environmental waters [26,38–40].

Polymeric SPE sorbents (Oasis HLB and Isolute ENV+), non-polar C_{18} and mixed polymeric and strong cation sorbent (Oasis MCX) cartridges were tested in one study [41]. As the study was performed at neutral pH, only for acidic compounds good recoveries were obtained, whereas basic and neutral compounds were poorly recovered using Oasis MCX cartridge. It could be explained that Oasis MCX is a mixed reversed phase-cation exchange cartridge and can efficiently extract acidic, basic and neutral compounds at low pH values, since the cation exchanger finds the basic compounds and the reversed phase can retain both acidic and neutral ones. In order to extract efficiently basic analytes, the samples should have been adjusted at low pH values. Polymeric sorbent Isolute ENV+ was effective only for few compounds as it is recommended for very polar organic compounds that are not retained on C_8 or C_{18} phases. However it can also retain neutral compounds at neutral pH (including MLs) through hydrophobic interactions. Non-polar C_{18} sorbent provided good results for the majority of the compounds. However, comparing the polymeric sorbent Oasis HLB to the other cartridges, Oasis HLB cartridges were much more efficient, yielding high recoveries for all target compounds. This sorbent can extract acidic, neutral and basic analytes at a wide range of pHs, including neutral pH. For this reason the sorbent can be suitable for the extraction of analytes when no sample pH adjustment is done.

Another option how to improve recovery and cleaning step during SPE is using tandem of two cartridges with different properties. Two kinds of cartridges were used for the extraction of many compounds from class of MLs, FQs, TCs, and SAs [42,43]. The first mentioned study used Oasis HLB cartridge on the top of SDB cartridge, the second study used the tandem Oasis HLB and MCX cartridges. Common procedure was that cartridges were conditioned separately, then connected and sample was passed through. The elution was done separately and the eluates combined and evaporated to the dryness. The tandem Oasis HLB and strong anion-exchange cartridges (SAX) has been employed for the determination of TCs, SAs and TMP in wastewaters [18]. The pH of sample was acidified to 2.5. As a result, the neutral and cationic forms of selected antibiotics were not retained on the SAX cartridge while humic acids and highly negatively charged organic matters were retained there. This improved further retention of the target antibiotics on Oasis HLB cartridges and following LC–MS analysis, where elevated baseline and severe matrix interferences were not shown as in case when only Oasis HLB catridges were used for the extraction. The elution of SAX yielded negligible amounts of antibiotics as well, confirming that the antibiotics were not retained on these cartridges. In addition, a visual inspection of SAX cartridges after the sample percolation showed that a significant amount of organic matter had accumulated in the sorbent.

The overview of extraction procedures used in multiresidue methods is shown in Table 1. In majority studies a washing step after sample percolation was performed. However, some authors do not refer washing step. This step is important in environmental area for washing impurities before the elution step. It is due to the following elution step when impurities can be eluted together with desirable analytes. The elution of cartridges is usually done by organic solvents such as MeOH (in majority of studies), acidified MeOH [4,19] or acetonitrile (ACN) [44–46]. The elution of cation-exchange cartridges is done by the mixture of MeOH and ammonia according to the specific sorbent and guidelines from manufacturer. Then the sample extracts are evaporated to dryness under a gentle stream of nitrogen and redissolved in mobile phase or in an appropriate solvent. They are stored in amber vials or dark glass to prevent photodegradation, especially of TCs, until injection onto the chromatographic system.

Only one study refers the use of Oasis HLB cartridges for SPE repeatedly [47]. After sample percolation and elution with methanol, the cartridge was rinsed with 10 mL of ACN and was

reused again. The same cartridge was used for extraction of 10 real samples or 20 standards.

Sample preparation step can be done through the on-line SPE connection or separately in off-line SPE configuration. For the determination of FQs, comparison of off-line and on-line SPE based on the use of C_{18} and SAX sorbents, respectively, for the preconcentration and clean-up steps has been performed [11]. Both SPE extractions were coupled to HPLC–UV. In both cases the degree of preconcentration and clean-up achieved was very high, allowing the use of UV detector for the identification and quantification of the studied FQs by HPLC. The limits of detection (LODs) were almost the same for off-line SPE as for on-line SPE. SPE with LC–MS through the on-line connection has been used for the determination of SAs and TCs in wastewaters [48]. Coupling of SPE procedures on-line with LC provided several advantages, such as reduction of the number of sample handling steps required, elimination the target loss by keeping in the cartridge from drying which results in recovery improvement and saving of the analytical time and minimization of the consumption of organic solvents for each analysis.

SPME can be another option for the extraction of aqueous samples [29,34,35]. SPME is an extraction technique that uses a fused-silica fibre with a solid stationary phase that collects analytes of interest. The technique is based on the partitioning of the analyte between stationary phase and matrix. Upon exposure to a sample, sorption of compounds to the solid phase occurs, resulting in simultaneous extraction, clean-up, and pre-concentration. After equilibration, adsorbed analytes are desorbed into an organic solvent, followed by chromatographic analysis. Typically, SPME method development requires the optimization of the equilibration conditions for each compound which can make the development more difficult. SPME showed some advantages over SPE such as decreased sample volume, ease and efficiency of sample processing and extraction and in some cases better elimination of matrix effects, although matrix effects are highly dependent on the type of the sample. Concerning the cost, in SPME individual fibre can be used for multiple extractions and very little organic solvent is required. In contrast, SPE cartridges are one-time use only, significantly more solvent is necessary and high volumes of samples are percolated. However, regarding to the sensitivity and precision, SPE was found more sensitive and it showed better accuracy during extraction as well [29,34] which showed the preferable use of SPE for the extraction of complex matrices.

In-tube SPME coupled with LC–MS/MS was employed for the determination of five FQs in environmental waters [35]. The in-tube SPME, a technique using an open tubular fused-silica capillary with an inner surface coating as extraction device, is simple and can easily be coupled on-line with HPLC, LC/MS and LC/MS/MS. In-tube SPME allows convenient automation of the extraction process, which not only reduces the analysis time over SPE, but also can provide better accuracy, precision, and sensitivity than off-line manual techniques. Small amount of sample (1 mL in this study) was extracted without any pretreatment and analytes were easily desorbed from the capillary by passage of the mobile phase with good recoveries. This method showed higher sensitivity than the direct injection method, because the compounds in the sample solution were preconcentrated in the capillary column during draw/eject cycles. However, this type of sample extraction is demanding special instrumentation and experiences.

There was one study that used lyophilization, because it was fast and consumption of organic solvent was very low. It was used in combination with SPE to pre-concentrate SAs [49].

3.1. Multiresidual methods

The overview of extraction procedures in multiresidue methods is shown in Table 1. These methods usually deal with the antibiotics from the different classes and with pharmaceuticals in some cases as well. In majority of methods the sample pH was adjusted to acidic values in order to obtain required chemical form of analytes. In some cases, pH was adjusted to high values, especially when MLs were included [22,23,25–27].

For the preconcentration and clean-up Oasis HLB columns were employed in majority of studies. It is due to their properties enabling retention of wide spectrum of different compounds achieving their good recoveries. However, other types of sorbents were utilized as well as can be seen in Table 1. In three studies the tandem of two SPE cartridges was used to achieve better clean-up step and thus high recoveries [19,42,43]. Washing step was referred in majority of studies as is suitable for extraction of analytes from the complex matrices as wastewaters.

3.2. Quinolone antibiotics

The structure of molecules of Qs contains carboxylic group which makes all these compounds acidic. In addition, the second-generation FQs have an amino group in the heterocyclic ring (namely piperazinyl) (Fig. 1(a)). Thus, Qs can be divided into the two groups according to the acid–base properties: acidic and piperazinyl quinolones with the heterocyclic group. Acidic quinolones have only one pK_a in range between 6.0 and 6.9. In acidic conditions they are in neutral form. In contrast, piperazinyl quinolones have two dissociation constants. The reported values of pK_1 and pK_2 are in the 5.5–6.3 and 7.6–8.5 range, respectively and thus, the intermediate form is a zwitterion. At acidic conditions they are in cationic form, which is important for their retention during the extraction. At basic conditions, the anionic species of both acidic and piperazinylic quinolones are less retained in comparison to cationic, zwitterionic and neutral species on the polymeric Oasis HBL column, but they may be retained on SAX cartridge. However, the different behaviour between both groups is observed at acidic pH [11,50]. The behaviour of Qs during SPE extraction was studied [36]. At acidic pH, the acidic quinolones, present as uncharged species in solution, were less retained on C_{18} cartridge. However, the piperazinyl quinolones present in cationic form at acidic pH were retained well. It is important to take this fact into account as typically sample pH is adjusted to very acidic values, far from the pK_a of the molecules, in order to ensure that they will be in the desired chemical form. However, very acidic pH may not be optimum for the preconcentration purposes [36]. Another possibility is the use of cation-exchange mechanism to retain the piperazinyl quinolones over a wide range of pH values. FQs can be extracted using cation-exchange sorbents when they are in cationic form which means the pH of sample has to be below their pK_a constants (2 units below the pK_a constants ≤ 3.0) [51,52].

The overview of procedures used during the sample preparation for Qs is showed in Table 2. During the pretreatment of sample the most of authors adjust the pH of sample in the range 2.8–4.0 or 4.5 to convert the FQs into a cationic form. The best pH value to assure that FQs are in cationic form should be two units below the pK_a constants. Only in one study, pH of water sample was adjusted pH 5.5 [50].

Following step in the sample preparation of Qs is an addition of chelator EDTA. FQs can be bound to divalent cations and thus could not be effectively retained on SPE cartridges and determined. However, the addition of EDTA is more important for the preparation of soil samples than water samples in the environmental analysis.

The mostly used SPE cartridges were polymeric Oasis HBL [50,53,54]. However, other types including cation-exchange [51,52], C_{18} [36] and Chromabond tetracycline [55] were used. A tandem system using an anion-exchange cartridge on the top together with Oasis HLB was also described [53]. The anion-exchange column was used for pre-purification since humic acids and others

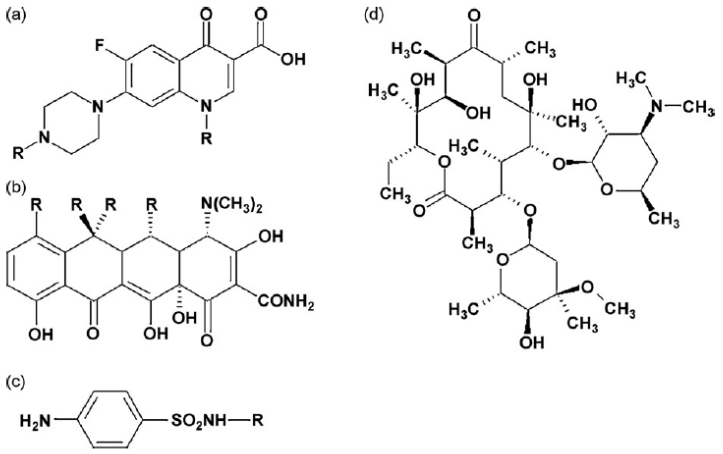

Fig. 1. General structures of selected groups of antibiotics included in this review: (a) fluoroquinolone, (b) tetracycline, (c) sulfonamide antibiotics, and (d) macrolide antibiotic erythromycin.

impurities were retained. Thus less impurity got onto Oasis HLB cartridge improving the clean-up procedure. As a result, a better clean-up step with less interference during the following analysis was achieved.

Different sorbents (C_{18}, SDB, SDP-RPS and MPC) were evaluated for the preconcentration of FQs in surface water [36]. It was concluded that the use of C_{18} cartridges was preferable with the sample pH value 4. These results are in accordance with results of Ferdig et al. [56]. The best results were obtained with Chromabond Tetracycline C_{18} modified silica in spite of these columns being recommended for the preconcentration of residues of TCs. The results from this cartridge were closely followed by results obtained on Oasis HLB [55,56].

Not in all studies, a concentration factor was referred. In one study following analysis was performed by LC–UV and concentration factor referred was 1000 [36]. This is sufficient preconcentration of the analytes for this kind of detection.

3.3. Tetracycline antibiotics

Concerning TCs none of the study dealed only with this group of antibiotics. They were involved in multiresidue studies together with other antibiotics or even pharmaceuticals. Despite of this, short chapter dealing with their properties is included. TCs contain hydronaphatacene backbone in their structure composed of four fused rings (Fig. 1(b)). Various analogues differ primarily by the different substitution. TCs show three pK_a values of approximately 3, 7 and 9. Throughout the range of pH, TCs always possess a local charge, and they are zwitterionic in the approximate range of pH 3–9. Thus pH adjustment is very important step in the analysis of TCs. The pH is usually adjusted to value $\leq$3 to assume that TCs will be in cationic form which is important for their optimal extraction. The best it is to perform pH adjustment immediately before extraction because they are no longer stable in acidic media.

TCs also tend to form complexes with divalent metal ions. This complexation can prevent effective extraction and has an effect on the spectral characteristics of TCs. TCs are amphoteric and most of them have a strong tendency to be bound irreversibly to the silanol groups in silica based stationary phases resulting in peak tailing. Addition of EDTA was performed in almost all studies to prevent TCs to be bound to divalent ions and thus the recovery of extraction to be increased. Another option how to improve their recovery was using Oasis HLB cartridges. They do not contain silanol groups thus TCs cannot be bound there irreversibly and the use of Oasis HLB leads to high recoveries.

3.4. Sulfonamide antibiotics and trimethoprim

Table 3 is showing the recent procedures for SAs extraction from water samples in studies dealing only with the SAs determination. All of studies used Oasis HLB columns except one using cation-exchange sorbent [34]. SAs contain one basic amine group ($-NH_2$) and one acidic sulfonamide group ($-SO_2NH-$) (Fig. 1(c)). They are ampholytes with weakly basic and acidic characteristics. It is explained by the charge state of the SAs at the particular pH values because of their pK_a values. The pK_{a1} (2–2.5) and pK_{a2} (5–8) correspond to the protonation of the aniline group and deprotonation of the sulfonylamido group, respectively. Weakly basic characteristics arise from the nitrogen of the anilinic substituent which is able to gain a proton, designated for protonation during ionization step of mass spectrometric detection, whereas the acidic characteristics arise from the N–H linkage of the sulfoamidic group which is able to release proton under specific pH conditions. Thus SAs are positively charged at acidic conditions at pH 2, neutral between pH 2 and 5, and negatively charged at alkaline conditions at pH above 5. Only two studies adjusted pH of sample to pH 3.0 or 3.4 respectively [34,57] and in the rest of studies pH adjustment was not reported. This is not usual when SPE and the interaction between the analytes and the sorbent of SPE columns are pH dependent. The interaction with the cartridge material is stronger for analytes in uncharged forms. Mostly, the sample pH was adjusted to value about 3.0, in range 2.0–4.0 in multiresidue methods as it can be seen in Table 1. This step led to good recovery rates which showed that pH adjustment of sample was very important and it was in agreement with their pK_a values.

Only one method referred the addition of EDTA to water sample [58]. This step was of no importance because SAs do not form complexes with divalent and polyvalent cations. Thus, during sample preparation for SAs determination it was not necessary to add EDTA.

As was already said above, majority of studies for the determination of SAs used Oasis HLB columns for their extraction from water samples (Table 3). All methods referred washing step after sample percolation through the SPE columns which was suitable in the

environmental analysis to remove interferences. In all cases water was used, except one study where 5% MeOH was used [3]. Elution of cartridges was done by organic solvent, but 5% MeOH was so weak that it did not manage to elute desirable analytes and thus can be used during the washing step.

Concerning the concentration factors, in majority of studies MS/MS detection was used. As it is very sensitive technique high concentration factor is not necessary. However in one study UV detection was used for the SAs and concentration factor was only 300. This method was applied for the detection of SAs in swine wastewater and as the UV detection was not very sensitive one could doubt if this preconcentration was satisfactory [64].

3.5. Macrolide antibiotics

Macrolide antibiotics contain a basic dimethylamine [$-N(CH_3)_2$] group, which is able to gain a proton (Fig. 1(d)). Thus, according to their chemical structure, MLs are basic compounds with pK_a values around 8. It was shown that their retention on reversed-phase cartridges was not pH dependent in the range 3–7 [9]. It should be noted that, in some cases, acidic pH values can promote the degradation of ERY. At pH bellow 7.0, ERY is immediately converted into its main degradation product ERY-H_2O and ERY is not detected in its original form but as a degradation product (ERY-H_2O) with an apparent loss of one molecule of water. Thus, ERY-H_2O is very often quantified in many studies [59], assuming that ERY is totally converted into ERY-H_2O in SPE procedures. Additionally, since the oral administration of ERY has to pass through strongly acidic conditions in the stomach, the degraded product ERY-H_2O, does not exhibit the original antibiotic properties [30].

Only four studies deal with the determination of MLs alone (Table 4). Considering the recoveries of the MLs, and the sensitivity and selectivity for ERY-H_2O in the acidic elution gradient, pH of sample was adjusted to higher values in range 5.0–7.0. Abuin et al. [17] showed the non-dependence of MLs in the range of pH 6–8. Addition of EDTA was performed in three studies. It is recommended as it was shown that MLs can bind divalent and polyvalent cations although they bind not so strong as TCs or FQs. The extraction was performed with polymeric Oasis HLB columns except one study using LiChrolute RP-18 sorbent [5].

An overview of extraction methods is in Table 4. The washing step was performed with water in three studies. The other study does not report the washing step although WWTPs effluents were analyzed [5]. It can be seen from recovery achieved that washing step was necessary. It can be due to the subsequent analysis by HPLC–MS because ESI source is highly susceptible to matrix interferences and thus some matrix effects could occur.

In case of MLs it can be shown that using sensitive LC–MS/MS detection, smaller amount of sample can be used for analysis of surface water samples, e.g. 250 mL [17] or 120 mL [30].

4. Analytical methods

There is a need for the development of analytical methods for sensitive and selective identification and quantification of antibiotics as environmental contaminations. LC–MS/MS is indicated as the technique of choice to assay relatively polar pharmaceuticals and their metabolites as it is especially suitable for the environmental analysis because of its selectivity comparing to UV [11,36] or FD [50,53,57].

In general, LC–MS can be used for quantitative purposes only when the analyte is present in simple matrices, such as tap water and bottled water, whereas LC–MS/MS is required for quantitation with simultaneous confirmation of identity of residues in complex matrices such as wastewaters [60].

4.1. Quantification by LC–MS/MS

In majority studies MS detection is used to identify and to quantify the substance or it can be used to confirm its molecular structure (Table 5). LC–MS/MS is often applied using triple quadrupole analyser and selected reaction monitoring (SRM) mode. This mode allows compound confirmation and providing structural information. In MS/MS, the most intensive fragment ion from precursor ion is used for quantification (transition 1). A less sensitive secondary transition is used as the second criterion for the confirmation purposes (transition 2). This mode also improves the precision and sensitivity of the analysis but does not collect full scan data. This can limit the availability of full scan data that can be used not only to the identify target analytes but also to detect additional unknown compounds. This can be good in searching of stable metabolites of antibiotics in waters that can be detected instead of parent compounds. The first step in the tandem MS detection is the selection of the precursor ion. The protonated molecular ions $[M+H]^+$ are generally considered to be the best precursor ions as can be seen in Table 5, except macrolide antibiotic ERY.

4.1.1. Chromatography

Mostly, C_{18} analytical columns were used for the separation of analytes (Table 5). Only one multiresidue study used C_{12} analytical column [18] and in one study C_8 analytical column was used for the separation of FQs [43]. Typically, mixtures of ACN or MeOH with water were used as mobile phases for the LC separation. Gradient elution was used in all multiresidue studies reported. In attempt to improve the ionization of analytes and sensitivity of MS detection in the analysis of antibiotics, modification of mobile phase was usually performed and has been accomplished with volatile additives such as formic acid (FAc), acetic acid (AcAc), and ammonium acetate (AmAc) at different concentration. Babić et al. [12] used oxalic acid and ACN in mobile phase for the determination of antibiotics in wastewaters. This was possible due to UV detection and thus nonvolatile modificator could be used.

UHPLC was used for the determination of compounds from different classes of pharmaceuticals including 5 antibiotics (chromatograms are shown in Figs. 2 and 3) [1,16]. UHPLC is a modern technique, using columns packed with sub-2 μm particles, which enabled elution of sample components in much narrower, more concentrated bands, resulting in better chromatographic resolution and increased peak capacity through rapid elution from short column. The speed provided by the UHPLC system in comparison to conventional HPLC system using 5 μm particles was compared. The reduction of analysis time was substantial because of the low dead volume of the whole system allowing short equilibration times (less than 1 min between the end of the gradient and the next injection) and reducing therefore the unproductive parts of chromatogram.

4.1.2. Mass spectrometry – ionization

For LC–MS and LC–MS/MS analysis of pharmaceuticals, two ionization interfaces has been the most widely used due to their sensitivity and robustness. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) satisfied the requirements. They produce protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecules. Both techniques work at atmospheric pressure which is suitable for the connection with LC system. ESI as a soft ionization technique is preferred in antibiotic residue determination due to its higher sensitivity and better reproducibility, since it is particularly suitable for both polar and non-polar analytes and for thermally labile substances. Positive electrospray ionization (ESI^+) was often preferred when both positive and negative ionization were possible as it can be seen in Table 5. Many antibiotic compounds are nonvolatile with high molecular weights and they respond well in ESI^+ which makes LC–MS or LC–MS/MS the

Table 5
HPLC analytical methods in multiresidue studies for the determination of different groups of antibiotics together with pharmaceuticals.

Substances determined	Matrix sample preparation	Stationary phase, analytical column	Mobile phase	Detection	Precursor ions	Internal standard	Sensitivity LOD/LOQ	Ref.
CIPRO, DOXY, SMX, TMP, ERY (+28 pharmaceuticals)	Surface water, SPE	Acquity UPLC BEH C_{18} (100 mm × 1 mm, 1.7 μm) (22 °C)	Gradient elution, A: water + MeOH + 0.5% AcAc, B: MeOH + 0.5% AcAc	ESI^+, MS–MS, SRM	$[M+H]^+$	Phenacetin-ethoxy-1-^{13}C	LOD = 0.1–0.5 ng L^{-1}, LOQ = 0.5–1.5 ng L^{-1}	[1]
SMX, ERY (+13 pharmaceuticals)	Surface, drinking, ground water, SPE	XTerra RP-18 (100 mm × 2.1 mm, 3.5 μm) (35 °C)	Gradient elution, A: 2 mM AmAc/MeOH, B: 2 mM AmAc/water	ESI^+, MS–MS, SRM	$[M+H]^+$	SDT	LOQ = 10–13 ng L^{-1}	[2]
CIPRO, NOR, ENRO, SARA, PIP, OXO, FLU, MINO, OXY, TET, DEME, CTC, DOXY, STZ, SMR, SMZ, SMT, SCP, SMX, SDT, TMP, ERY, ROXI, TLS	Drinking water, SPE	Pursuit C-18 (150 mm × 2 mm, 3 μm)	Gradient elution, A: 0.1% FAc + water, B: ACN	ESI^+, MS–MS	$[M+H]^+$	Simatone, $^{13}C_6$-SMZ	LOD = 0.5–6 ng L^{-1}, LOQ = 1–32 ng L^{-1}	[4]
SDZ, STZ, SMZ, SPY, SMX, N^4-acetyl-SMX, TMP, AZI, CLAR, ERY, ROXI	WWTP effluent, SPE	YMC Pro C_{18} (150 mm × 2.0 mm, 3 μm) (30 °C)	Gradient elution, A: water + 1% FAc (pH 2.1), B: MeOH + 1% FAc	ESI^+, MS/MS, SRM	$[M+H]^+$	SMR, TLS, JOSA	LOQ = 1–214 ng L^{-1}	[7]
ERY, CLAR, ROXI (+hormones)	WWTP influents and effluents, SPE, SEC	Phenosphere-next RP_{18} (150 mm × 2.0 mm, 3 μm) (25 °C)	Gradient elution, A: 10 mM AmAc, B: ACN	APCI, MS/MS, SRM		(E)-9-[-O-methyloxime)]-ERY	LOQ = 2–6 ng L^{-1}	[8]
CIPRO, NOR, OFLO, DOXY, SMX, TMP (+β-lactams)	Hospital wastewater, SPE	YMC Hydrosphere C_{18} (150 mm × 4.6 mm, 5 μm) (25 °C)	Gradient elution, A: water + 0.1% FAc, B: ACN + 0.1% FAc	ESI^+, MS–MS, SIM	$[M+H]^+$	ENRO, DEME, SMZ, Diaverine	LOQ = 0.01–0.68 ng inj.	[10]
ENRO, OXY, SMZ, SDZ, SGN, TMP (+β-lactams)	Wastewater, SPE	LiChrospher 100CN (125 mm × 4.0 mm, 5 μm) (30 °C)	Gradient elution, A: 0.01 M oxalic acid, B: ACN	DAD, 280 nm			LOD = 0.1–40 μg L^{-1}, LOQ = 1.5–60 μg L^{-1}	[12]
SMX, TMP, ERY-H_2O (+27 pharmaceuticals)	Surface water, SPE	Synergi Max-RP C12 (250 mm × 4.6 mm, 4 μm)	Gradient elution, A: 0.1% FAc in water, B: MeOH	ESI^+, MS/MS	$[M+H]^+$, $[M+H-H_2O]^+$	$[^{13}C_1]$-ERY	LOD = 1 ng L^{-1}	[18]
CIPRO, ENRO, NOR, OFLO, SMZ, SMX, TMP	WWTP effluents (2nd, 3rd) SPE	Zorbax SB-C_{18} (150 mm × 2.1 mm, 5 μm) (30 °C)	Gradient elution, A: 1 mM AmAc + 0.007% AcAc + 10% ACN, B: ACN	ESI^+, MS, SIM	$[M+H]^+$	LOME, SMR	LOD = 20–90 ng $_{-1}$	[19]
OXY, CTC, MINO, DEME, MECLO, TET, DOXY, STZ, SMX, SMR, SCP, SMZ, SDT, ERY, ROXI, TLS	River water, SPE	Xterra MS C_{18} (50 × 2.1 mm, 2.5 μm) (15 °C) (TCs, SAs) (45 °C) (MLs)	Gradient elution, A: 0.1% FAc + water (pH 2.74), B: 0.1% FAc + ACN	ESI^+, MS–MS, SRM	$[M+H]^+$, $[M+H-H_2O]^+$	Simatone	LOQ = 10 ng L^{-1}	[21]
CIPRO, ENRO, NOR, OFLO, OXO, PIP, CTC, DOXY, OXY, TET, 16 SAs, CLAR, ERY, ROXI	WWTP final effluents SPE	Genesis C_{18} (150 mm × 2.1 mm, 3 μm)	Gradient elution, A: ACN, B: 20 mM AmAc (0.1% FAc, pH 4.0) (FQs, TCs, SAs), B: 20 mM AmAc (0.05% FAc, pH 4.0) (MLs)	ESI^+, MS–MS, SRM	$[M+H]^+$		LOD = 1–8 ng L^{-1}	[22]
CIPRO, CTC, TET, SDT, SMZ, SMT, SMX, STZ, SSX, CLAR (+20 pharmaceuticals)	WWTP influent and effluent, SPE	Lichrospher RP-18 (150 mm × 3 mm, 5 μm) (FQs, SAs, MLs), Genesis C_{18} (150 mm × 2.1 mm, 3 μm) (TCs)	Gradient elution, A: 0.1% FAc in water, B: 0.1% FAc in ACN	ESI^+, MS–MS			LOD = 0.42–8.11 ng L^{-1}	[23]

OFLO, NOR, CIPRO (+5 pharmaceuticals)	Groundwater, surface water, WWTP influent and effluent, SPE	Zorbax XDB-C_{18} (50 mm × 2.1 mm, 5 μm) (30 °C)	Gradient elution, A: 1% AcAc, B: ACN	ESI$^+$, MS–MS, SRM	$[M+H]^+$	ENRO	LOQ = 1.6–163 ng L^{-1}	[24]
CTC, DOXY, MECLO, OXY, TET, SCP, SDT, SMR, SMZ, STZ, TMP, ERY, ROXI, TLS (+pharmaceuticals)	Surface water, SPE	Genesis column (150 mm × 2.1 mm) (25 °C)	Gradient elution, A: 0.015% heptafluorobutyric acid + 0.5 mM AmAc + 25% MeOH, B: ACN	ESI$^+$, MS–MS, SRM		$^{13}C_6$-sulfamethazine phenyl	LOD = 30–600 ng L^{-1}, LOQ = 0.1–60 ng L^{-1}	[25]
SMX, TMP, ERY (+13 pharmaceuticals)	Surface water, wastewater, SPE	Luna C_{18} (250 mm × 10 mm, 10 μm)	Gradient elution, A: water, B: MeOH, C: 10 mM AmAc, D: 0.87 M AcAc	ESI$^+$, MS–MS, SRM			LOD = 0.07–0.13 ng L^{-1}, LOQ = 0.22–0.43 ng L^{-1}	[26]
TMP, ERY (+16 pharmaceuticals)	Hospital wastewaters, SPE	Purospher Star RP-18 (125 mm × 2.0 mm, 5 μm)	Gradient elution, A: ACN, B: 0.1% FAc	ESI$^+$, MS–MS, SRM	$[M+H]^+$	$^{13}C_3$-phenacetin, 2–4-Dd^5	LOD = 3.8–9.2 ng L^{-1}, LOQ = 11–26 ng L^{-1}	[27]
SMX, TMP (+22 pharmaceuticals)	Surface water, ground water, SPE	Metasil basic (150 mm × 2.0 mm, 3 μm)	Gradient elution, A: 10 mM ammonium-formate/FAc buffer (pH 3.7), B: ACN	ESI$^+$, MS, SIM		[^{13}C]-caffeine, [^{13}C]-phenacetin	LOD = 14–23 ng L^{-1}	[28]
SPY, SMX, SSX, SMZ, SDT, TMP, ERY, CLAR, ROXI	WWTP influent and effluent SPME, SPE	Zorbax Eclipse XDB C_{18} (250 mm × 4.6 mm, 5 μm), Ultra C18 (250 mm × 4.6 mm, 5 μm)	Gradient elution, A: ACN, B: 10 mM AmAc/0.1% FAc/10% ACN	ESI$^+$, MS–MS, SRM		$^{13}C_6$-SMZ, JOSA	LOD_{SPE} = 0.08–6.1 ng L^{-1}, LOQ_{SPE} = 0.27–20 ng L^{-1}, LOD_{SPME} = 2.8–410 ng L^{-1}, LOQ_{SPME} = 9.2–1380 ng L^{-1}	[29]
OXY, CTC, TET, DEME, DOXY, MECL, MINO, STZ, SMR, SMZ, SCP, SMX, SDT	Surface waters, SPE	XTerra MS C_{18} (50 mm × 2.1 mm, 2.5 μm) (15 °C)	Gradient elution, A: water + 0.1% FAc, B: ACN + 0.1% FAc	UV, 360 nm (TCS), 260 nm (SAs) ESI$^+$, MS–MS, SRM	$[M+H]^+$	Simatone	LOD = 30–50 ng L^{-1}	[32]
CTC, DEME, DOXY, MECL, OXY, TET, STZ, SMZ, SCP, SMY, SDT	WWTP influent and effluent, SPE	XTerra MS C_{18} (50 mm × 2.1 mm, 2.5 μm) (15 °C)	Gradient elution, A: water + 0.1% FAc, B: ACN + 0.1% FAc	ESI$^+$, MS–MS, SRM	$[M+H]^+$	Simatone	LOD_{INF} = 40–70 ng L^{-1}, LOD_{EFF} = 30–50 ng L^{-1}	[33]
SMX, acetyl-SMX, TMP, ERY (+13 pharm)	Surface water, sewage effluents, SPE	C_{18} Luna (250 mm × 2 mm, 5 μm)	Gradient elution, A: 40 mM AmAc (pH 5.5 by FAc), B: MeOH, C: water	ESI$^+$, MS/MS, SRM	$[M+H]^+$	[^{13}C]-phenacetin	LOD = 10–50 ng L^{-1}	[38,40]
SMX, TMP (+20 pharmaceuticals)	WWTP influent and effluent, SPE	Sunfire C_{18} (150 mm × 4.6 mm, 3.5 μm), Sunfire C_{18} narrow bore (50 mm × 2.1 mm, 3.55 μm)	Gradient elution, A: 0.1% AmAc in water (pH 6.2), B: 0.1% AmAc in ACN, A: 20:80 ACN:water + 0.1% AmAc, B: 80:20 ACN:water + 0.1% AmAc	UV, 270 nm, ESI$^-$$_{SMX}$, ESI$^+$$_{TMP}$, MS–MS	$[M-H]^-$, $[M+H]^+$		$LOD_{influent}$ = 72–171 ng L^{-1}, $LOQ_{influent}$ = 241–570 ng L^{-1}, $LOD_{effluent}$ = 20–166 ng L^{-1}, $LOQ_{effluent}$ = 67–553 ng L^{-1}	[39]
OFLO, SMX, TMP, ERY, AZI (+27 pharmaceuticals)	Ground waters, WWTP influents and effluents, SPE	Purospher Star RP-18 (125 mm × 2.0 mm, 5 μm)	Gradient elution, A: ACN + MeOH (2:1), B: 5 mM AmAc/AcAc buffer, pH 4.7	ESI$^+$, MS–MS, SRM	$[M+H]^+$	$^{13}C_3$-phenacetin	LOD = 1–43 ng L^{-1}, LOQ = 3–120 ng L^{-1}	[41]
CIPRO, OFLO, CTC, DOXY, OXY, TET, SDM, SMX, TMP, AZI, CLIN, CLAR, ERY, ROXI, SPIR, TLS, VAN (+β-lactams)	Surface water, SPE	Phenomenex SYNERGI Hydro-RP C18, 4 μm	Gradient elution not specified	ESI$^+$, MS–MS	$[M+H]^+$		LOQ = 0.5–30 ng L^{-1}	[42]

Table 5 (*Continued*)

Substances determined	Matrix sample preparation	Stationary phase, analytical column	Mobile phase	Detection	Precursor ions	Internal standard	Sensitivity LOD/LOQ	Ref.
CIPRO, ENRO, NOR, SARA CTC, DOXY, OXY, TET, SMT, STZ, SMR, SMZ, SCP, SMX, SDT, TMP, ERY-H_2O, ROXI, TLS	Ground water, WWTP influent and effluent, SPE	Luna C_8 (150 mm × 3.0 mm, 3 μm) (FQs), MS Xterra C_{18} (150 mm × 3.0 mm, 3.5 μm) (TCs), Luna phenylhexyl (150 mm × 3.0 mm, 3.5 μm) (SAs, MLs)	Gradient elution, A: 20 mM ammonium-formate + 0.3% FAc + MeOH, B: MeOH + 20 mM ammonium-formate + 0.5% FAc, Gradient elution, A: 20 mM AmAc (pH 6.5) + ACN, B: 20/80 A/ACN (SAs, MLs)	ESI$^+$, MS	$[M+H]^+$	$^{13}C_6$-SMZ, [^{13}C]-ERY-H_2O, MECLO, terbuthylazine	LOQ_{SW} = 10–50 ng L^{-1}, LOQ_{WWTP} = 50–100 ng L^{-1}	[43]
CIPRO, ENRO, TET, OXY, CTC, SMZ, SDT, SMX, TMP, CLIN, ERY, ROXI, TLS	Surface, ground water, WWTP effluent, SPE	Beta-Basic-18 C_{18} (100 mm × 2.1 mm, 3 μm) (30 °C)	Gradient elution, A: ACN, B: MeOH, C: water + 0.3% FAc	ESI$^+$, MS-MS, SRM	$[M+H]^+$	$^{13}C_6$-SMZ	LOD = 27–190 ng L^{-1}, LOQ = 100–650 ng L^{-1}	[44]
NOR, TET, TMP, ERY-H_2O (+β-lactams)	Surface water, SPE	XBridge C_{18} (50 mm × 2.1 mm, 5 μm)	Gradient elution, A: 0.01% FAc in water, B: MeOH	ESI$^+$, MS–MS			LOD = 2–13 ng L^{-1}	[45]
OXO, SDZ, TMP	Surface water, SPE	Luna C_{18} (150 mm × 4.6 mm, 5 μm) (25 °C)	Gradient elution, A: 10% (v/v) MeOH + 0.1% FAc, B: 90% (v/v) MeOH + 0.1% FAc	APCI$^+$, MS–MS, SRM	$[M+H]^+$		LOD = 1–2 ng L^{-1}	[46]
CIPRO, ENRO, TET, OXY, DOXY, CTC	River water, WWTP influent and effluent, SPE	Kromasil 100 C_{18} (250 mm × 4.6 mm, 5 μm) (35 °C)	Gradient elution, A: water + 1% AcAc (pH 2.8), B: ACN	ESI$^+$, MS, SIM	$[M+H]^+$		LOD = 4–6 ng L^{-1}	[47]
OXY, MINO, DOXY, DEME, MECLO, CTC, TET, SDT, SMX, STZ, SCP, SMR, SMZ, SMM	Surface water, wastewater, on-line SPE	Atlantis dC_{18} (50 mm × 2.1 mm, 3 μm)	Gradient elution, A: 0.1% FAc in water, B: 0.1% FAc in ACN	DAD, API$^+$, MS	$[M+H]^+$	Simatone	LOD = 30–110 ng L^{-1}	[48]
CIPRO, NOR, OFLO, DOXY, SMX, TMP, ERY-H_2O (+β-lactams)	WWTP effluent, SPE	YMC Hydrosphere C_{18} (150 mm × 4.6 mm, 5 μm) (25 °C)	Gradient elution, A: water + 0.1% FAc, B: ACN + 0.1% FAc	ESI$^+$, MS–MS, SRM	$[M+H]^+$, $[M+H-H_2O]^+$	ENRO, DEME, SMZ, Diaverine	LOQ = 6–160 ng L^{-1}	[59]
OFLO, SMX, TMP, AZI, ERY (+29 pharmaceuticals)	River water, WWTP influent and effluent, SPE	Acquity UPLC BEH C_{18} (50 mm × 2.1 mm, 1.7 μm)	Gradient elution, A: 5 mM AmAc/AcAc (pH 4.8) B: ACN + MeOH (2:1)	ESI$^+$, MS–MS, SRM	$[M+H]^+$	^{13}C-phenacetin	$LOD_{influent}$ = 10–500 ng L^{-1}	[61]
CIPRO, NOR, LOME, LEV, GAT, SPAR, MOXI, SMX, TMP (β-lactams)	Surface water, SPE	Inertsil ODS-3V C_{18} (250 mm × 4.6 mm, 5 μm)	Gradient elution, A: 0.1% TFA, B: ACN	PDA, 280 nm, ESI$^+$, MS, SIM	$[M+H]^+$		LOD = 0.6–8.1 μg L^{-1}, LOQ = 2.7–24 μg L^{-1}	[66]
ROXI (+novobicin, atorvastatin)	Surface water, WWTP effluent, SPE	Microbore column YMC ODS-AQ (100 mm × 1.0 mm, 3 μm)	Gradient elution, A: ACN, B: 10 mM AmAc	ESI$^+$, MS/MS, SRM	$[M+H]^+$		IDL = 3 pg	[67]
TLS (3 veterinary antibiotics)	Surface water, SPE	Thermo Hypersil Gold (50 mm × 2.1 mm, 5 μm)	Gradient elution, A: ACN + 20 mM heptafluorobutyric acid, B: water + 20 mM heptafluorobutyric acid	ESI$^+$, MS–MS, SRM	$[M+H]^+$		IQL = 10 g L^{-1}, LOQ = 35 ng L^{-1}	[68]
OXO, NAL, FLU, MAR, OFLO, ENRO, PEFL, CIPRO, PIP, NOR (+β-lactams)	Groud and surface water, on-line SPE	Kromasil C-18 (100 mm × 2.1 mm, 5 μm)	Gradient elution, A: water + 0.1% FAc, B: MeOH + 0.1% FAc	ESI$^+$, MS–MS, SRM	$[M+H]^+$			[69]

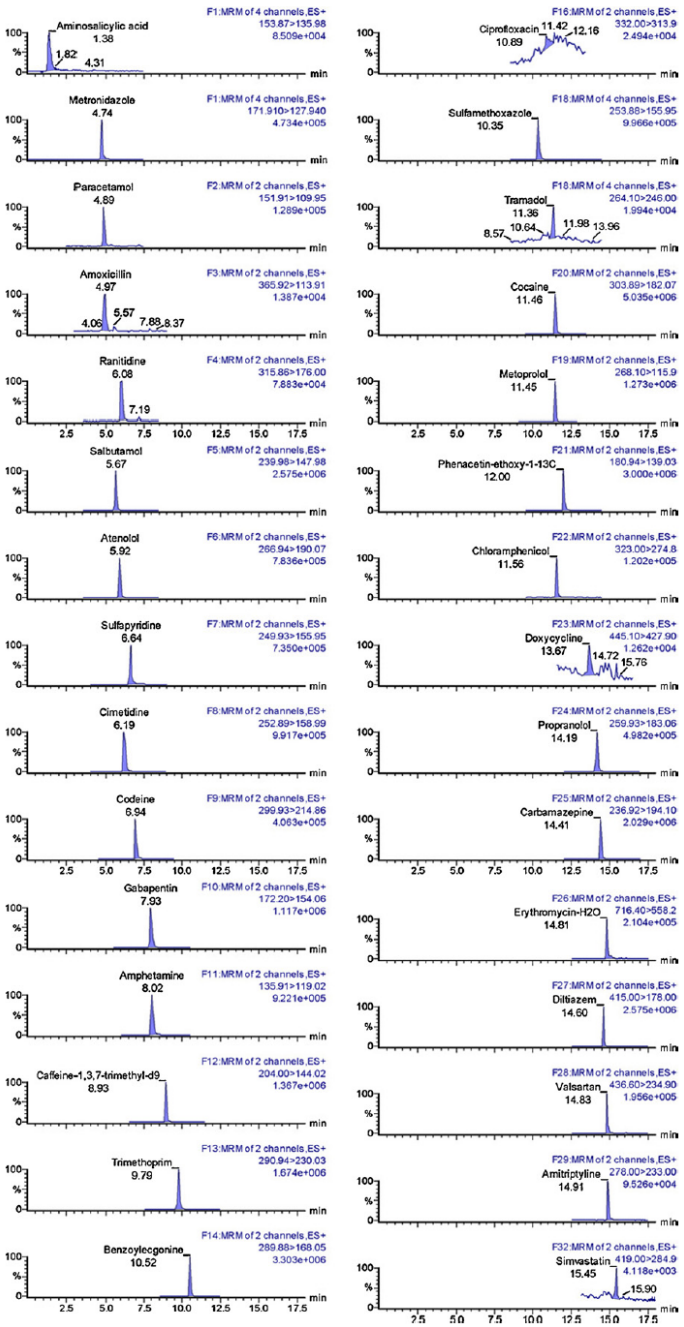

Fig. 2. UHPLC–MS/MS separations for chosen pharmaceuticals spiked into water and extracted by SPE (concentration of pharmaceuticals, 100 ng L^{-1}; IS, 200 ng L^{-1}) (antibiotics included – CIPRO, SMX, DOXY, ERY-H_2O, TMP). Reprinted from [1] - copyright Elsevier.

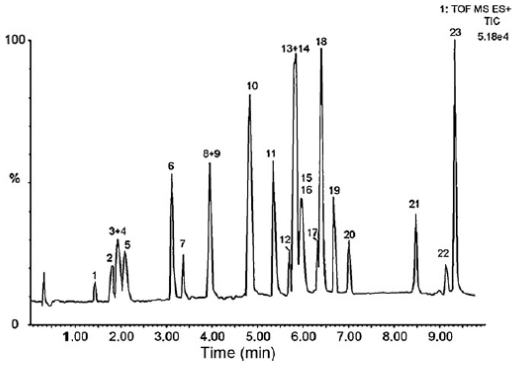

Fig. 3. UPLC–TOF total ion chromatogram showing the separation of 23 pharmaceutical compounds (analgesics, beta-blockers, lipid regulators, antiulcer agents, antihistaminicsm, psychiatric drugs and antibiotics ERY, AZI, SMX, TMP, OFLO) analyzed in positive ion mode (100 ng mL^{-1} standard solution). Reprinted from [61] - copyright Elsevier.

technique of choice for their separation and analysis. Only in two multiresidue studies APCI ionization technique is referred in comparison to the rest of studies using ESI for ionization of analytes [8,46].

The performance of APCI and ESI in the analysis of diverse drugs was compared. ESI was found to provide the best LODs of MLs in influents and effluents of WWTPs [8]. However, due to the matrix effects in LC–MS/MS it was concluded that APCI mode should be preferred than ESI, even though less sensitivity is obtained in standard solutions. Also, the addition of AmAc to the mobile phase for the separation of MLs increased the ionization performance. Stolker et al. [2] have referred 10-fold higher sensitivity for the most of pharmaceuticals in ESI mode than in APCI mode in his study which was according to results of Schlüsener and Bester [8]. Both, positive and negative ionization modes were evaluated for the analysis of different groups of antibiotics (TCs, MLs, SAs, TMP) [25]. TCs and MLs, in general, can be easily protonated and analyzed in positive ion mode. SAs can be readily detected in both negative and positive ESI modes.

4.1.3. Matrix effects

In LC–MS analysis, especially in the environmental analysis, the signal intensity of antibiotics may be considerably suppressed in wastewater matrices. Matrix effects occur very often in ESI MS analysis. It is the main disadvantage of ESI MS, because ESI source is highly susceptible to other components present in the matrix. As a result, signal suppression leads to erroneous results. The decrease of method sensitivity can be caused by several factors. Firstly, the antibiotics may sorb to organic matter in the samples which causes that sample preparation step is not effective in the point of view concentration of antibiotics and those are more difficult to detect. Secondly, contaminants in the sample matrix may interfere with the analyte peaks by raising the chromatogram baseline. Thirdly, contaminants may reduce the ionization efficiency of the analytes by taking up some of the limited number of excess charged sites on the surfaces of electrospray droplets [1,18,41]. Previously cited work studied the signal suppression [18]. The observation suggested that comparison of signal suppression among the antibiotics indicated that antibiotics within the same class generally exhibited a similar degree of signal suppression (FQs *vs.* SAs *vs.* TMP were compared). It was concluded that FQs were more susceptible to signal suppression than SAs and TMP. Signal suppression may be minimized by improving selective extraction, effective sample clean-up procedures after the extraction, improvement of the chromatographic separation and quantification by internal standards or standard addition method.

Another complication encountered in the LC–MS analysis is that retention times of antibiotics might drift significantly. One study refers observation of drift up to 2 min in some wastewater samples for FQs and TMP [18]. However, the molecular and confirming ions provided enough evidence to identify the antibiotics in these cases. This was solved by adjusting the eluent buffer concentration to minimize the drift in retention time and improve the peak shape. On the other hand, increasing the buffer concentration can reduce signal intensity.

4.1.4. Internal standard

For the environmental analysis it is suitable to use internal standard (IS) to improve accuracy of quantitation. It is necessary to choose the compound which does not occur in the environment to avoid false positive results. IS could be used as surrogate standards—they are added prior to the enrichment to assess possible losses during the analytical procedure or like instrumental standards that are added to the final extracts prior to measurement. While the surrogate standards are used for the quantification, the instrumental standards are used to check the instrument performance during measurement [7].

In many multiresidue studies, simatone was chosen for MS detection as IS because it is eluted within the same chromatographic time frame as analytes, responded well in ESI$^+$ mode and had no noticeable matrix effects [4,21,32,33,48]. ^{13}C-phenacetin was used very often in studies where antibiotics were analyzed together with other pharmaceuticals [1,24,27,38,40,41,61]. Although simatone or phenacetin are good ISs, it is desirable that both IS and target compounds should have the structural similarities in order to reflect the properties of the target compound during the entire analytical procedure. This indicates that it would be more desirable to have an IS (e.g., an isotopically labelled compounds, a structurally similar compound) for each individual antibiotic or at least for each class of antibiotics. In an attempt to represent the analytes most effectively, carbon-13- and deuterium-labelled surrogate were used as ISs (when commercially available, because only a limited number of isotopically labelled antibiotic substances are currently commercially available). This will certainly play an important role in further improvement of ease and accuracy of the determination of antibiotics in the environmental samples. It should be noted that a labelled standard will only be effective if it perfectly matches the target analyte(s) in terms of chromatographic retention time. Therefore a structurally identical isotopically labelled IS should ideally be added for every analyte to be determined [10]. Some compounds from the same class of antibiotics which are not used in human therapy can be employed as IS because they are not expected at significant concentration in municipal wastewaters.

4.2. Screening methods

Screening analytical methods can be based on the strategy of screening and confirmation. Screening methods are methods that are used to detect the presence of an analyte or class of analytes at the level of interest. Confirmatory methods are methods that provide full or complementary information enabling the analyte to be identified unequivocally at the level of interest [2].

Radioassay has also been reported as a screening method for the detection of antibiotics; however its low selectivity allows only semi-quantitative results [31]. Immunoassay is a next method of analysis that relies on specific interactions between antibodies and antigens to measure a variety of substances. Qualitative, semi-quantitative or quantitative immunoassay techniques are based on

the fundamental concept that antibodies prepared in animals can recognize and bind with high specificity to the antigen that stimulated their production. Immunoassays are an appropriate method of analysis in cases where real-time data are critical, field assays are needed and most samples are expected to be negative. Therefore, Immunoassays are well-suited for large-scale surveys and monitoring programs where sophisticated laboratory capability is not available [31]. Radioimmunoassays (RIAs) have been used as both qualitative or semi-quantitative tool and a screening method to reduce sample load for direct analyses. RIAs generally employ radiolabelled antigens and specific antibodies for the quantitative detection of antigens such as drugs and hormones. Yang and Carlson [31] assessed the applicability of SPE/RIA method for the routine monitoring of antibiotics in water and wastewater sources. The efficiency between SPE/RIA and SPE/LC–MS was compared for determination of TCs and SAs in water samples.

4.3. Multiresidue methods

Table 5 refers the recently published methods dealing with the determination of antibiotics from different classes even together with some pharmaceuticals. LC–MS or LC–MS/MS was used in majority of studies. One study referred UV detection of antibiotics in wastewaters [12]. However, in some studies, UV detection was used during the development step; MS detection was employed for further quantification and confirmation of analytes. ESI^+ was chosen for the ionization in all studies, except two studies using APCI [8,46].

For separation, C_{18} analytical columns were mostly used. However, in some studies, C_8 [43], C_{12} [18] and phenylhexyl [43] analytical columns were employed. The gradient elution mode was utilized in all studies.

Two works used UHPLC in tandem with MS [1,61] using C_{18} analytical columns with 1.7 μm particles.

4.4. Fluoroquinolone antibiotics

FQs are widely used in human medicine. The methods deal mostly with norfloxacin (NOR), ciprofloxacin (CIPRO) and ofloxacin (OFLO) which are intended for humans use. Concerning the veterinary compounds, the most studied was enrofloxacin (ENRO) and sarafloxacin (SARA). An overview of methods dealing with the determination of Qs and FQs in environmental waters is shown Table 6. LC coupled to tandem MS method is nowadays a technique of choice for the determination of very low concentrations of FQs in environmental waters because of its selectivity and specificity. However, LC with FD was used in six works [50–55]. FQs are naturally highly fluorescent compounds and RP-LC–FD can be used for their determination. They were monitored at excitation wavelength 278 nm and emission wavelength 450 nm. Nevertheless, some studies used different wavelengths for each compound [50,55]. UV detection was also reported for the determination of FQs [36] but it was not as specific as FD. The detection was performed at 275 nm [36] or at 255 nm [36] depending on group of the compounds studied.

LC–MS/MS methods employed ESI^+ in all studies. For the quantitation and confirmation, SRM experiments were used [35,52,55]. In some studies single MS was utilized together with ESI^+ and SIM (selected ion monitoring) experiment [51,55,66]. Protonated molecule peak $[M+H]^+$ was used as a precursor ion for the quantitation.

Separation was performed on C_{18} stationary phase [36,55]. However, RP-amide C_{16} stationary phase [51] and C_8 analytical column were utilized [35,50,52]. A monolithic column was used for LC separation in two studies [53,54]. In four studies the separation was performed using gradient elution [36,50,51,55] and in four studies by isocratic elution [35,52–54]. Mobile phases contained MeOH or ACN or both together with volatile additive such as FAc or ammonium formate for the improvement of the ionization during MS analysis. Nonvolatile oxalic acid [50] and phosphoric acid [53,54] were added to mobile phases when FD was used.

Only one study used isotopically labelled CIPRO as IS [52]. Other studies did not refer using IS. In some multiresidue studies ENRO [10,24,59] or lomefloxacin [18] were used as ISs. These ISs were suitable because of similarity with the analyzed compounds, but there was a risk about their possible occurrence in the environment. ENRO can be used in veterinary medicine and thus can enter the environment leading to false results. For this reason it is better to use isotopically labelled ISs.

Capillary electrophoresis is another possible analytical technique that has scarcely been applied by environmental researches. This technique is less robust and less suitable for the routine monitoring antibiotics in environmental waters than HPLC. Capillary zone electrophoresis (CZE) with FD was employed for separation of 9 FQs in surface water samples [56]. CZE with UV detection was used for simultaneous determination of 5 FQs [62].

4.5. Tetracycline antibiotics

As it was already mentioned in the chapter extraction any of studies dealed only with the determination of TCs in environmental waters. TCs were determined only in multiresidue studies together with other antibiotics or even with other pharmaceuticals (Table 5). However, LC–MS/MS methods are nowadays technique of choice for the analysis of TCs in environmental waters. Some authors employed HPLC with UV detection. The detection was performed at 360 nm [32,48] or at 280 nm [11]. 280 nm was used for the detection in multiresidue study in which other veterinary antibiotics were determined, although UV detection was not as sensitive and specific as MS detection. FD can be used for detection of TCs if they are derivatized before.

Concerning MS detection for quantitation of TCs the protonated molecule $[M+H]^+$ can be chosen as a precursor ion. TCs exhibit characteristic fragment ions such as a neutral loss of 17, $[M+H\text{-}NH_3]^+$, and 35 amu, $[M+H\text{-}NH_3\text{-}H_2O]^+$ and 18 amu loss, $[M+H\text{-}H_2O]^+$ of tetracycline (TET) and oxytetracycline (OXY).

It is important that TCs strongly bind to silanol groups and consequently peak tailing could be observed. Using C_{18} end-capped column and an addition of 1% AcAc to aqueous solvent of mobile phase was employed to avoid tailing peaks during determination of TCs [47].

As it was already mentioned, the use of isotope labelled IS is the best approach for the quantification in environmental analysis. However using isotope labelled IS was not referred in any study. Some compounds from the class of TCs were also utilized as IS in multiresidue studies: demeclocycline [10,59] or meclocycline [3].

4.6. Sulfonamide antibiotics and trimethoprim

SAs belong to the most studied group of antibiotics. Among them, mostly SMX and TMP are studied due to their wide use in human medicine for the treatment of bacterial infections. From veterinary compounds, sulfathiazole (STZ), sulfamethazine (SMZ), sulfadiazine (SDZ) and sulfachloropyridazine (SCP) have been included in many studies dealing with their determination in environmental water samples. In most cases, SAs were not studied alone but their determination was made simultaneously with other antibiotics. They have been determined in different kinds of environmental waters mainly in surface waters and wastewater samples. SPE was preferred technique of sample preparation in most cases. The overview of methods dealing only with SAs determination is shown in Table 7.

Table 6
HPLC analytical methods for the determination of FQs.

Substances determined	Matrix sample preparation	Stationary phase, analytical column	Mobile phase	Detection	Precursor ions	Internal standard	Sensitivity LOD/LOQ	Ref.
ENO, OFLO, CIPRO, NOR, LOME	Surface water, wastewater SPME	CAPCELL PAK C_8 (100 mm × 2.1 mm, 5 μm) (40 °C)	5 mM ammonium-formate (pH 3) + ACN (85/15)	ESI^+ MS/MS SRM	$[M+H]^+$		LOD = 7–29 ng L^{-1}	[35]
CIPRO, DAN, ENO, ENRO, NOR, CINO, FLU, NAL, OXO	Surface water, SPE	Polarity dC_{18} (150 mm × 3 mm, 3 μm)	Gradient elution, A: FAc (pH 2.5), B: ACN	DAD, 275 or 255 nm			LOD = 8–20 ng L^{-1}	[36]
CIPRO, DAN, DIF, ENRO, FLU, MAR, NAL, NOR, OXO, SARA	Surface water, SPE	Inertsil C_8 (250 mm × 4.6 mm, 5 μm)	Gradient elution, A: 10 mM oxalic acid buffer (pH 4) + ACN (89:11), B: ACN	FD, 248–297 nm, 361–507 nm			LOD = 0.05–1 μg L^{-1}	[50]
CIPRO, DIF, ENRO, LOME, NOR, OFLO, PIP, SARA, TOS	WWTP effluents, surface water, SPE	YMC ODS-AQ S-3 (50 mm × 4.0 mm) (23 °C), Discovery RP-Amide C16 (50 mm × 4.0 mm)	Gradient elution, A: water (pH 3.0) + ACN (98:2), B: ACN	ESI^+, MS, SIM, FD, 278 nm, 445–500 nm	$[M+H]^+$		LOD_{MS} = 8.6–49 ng L^{-1}	[51]
OFLO, NOR, CIPRO	WWTP effluents, SPE	Zorbax SB-C_8 (150 mm × 2.1 mm, 3.5 μm)	ACN/MeOH/FAc/water (6/12/0.5/81.5)	FD (278, 450 nm), ESI^+, MS, SIM, ESI^+, MS/MS, SRM	$[M+H]^+$	CIPRO-$^{13}C_3$ ^{15}N	LOQ = 2–10 ng L^{-1}	[52]
OFLO, NOR, CIPRO, ENRO	Wastewater, SPE	Chromolith Performance RP-18e (100 mm × 4.6 mm)	0.025 M H_3PO_4 (pH 3.0 by TBA) + MeOH + ACN (920/70/10)	FD, 278 nm, 450 nm			LOD = 8.5–85 ng L^{-1}, LOQ = 25–250 ng L^{-1}	[53]
NOR, CIPRO, ENRO	Surface water, SPE	Chromolith Performance RP-18e (100 mm × 4.6 mm)	0.025 M H_3PO_4 (pH 3.0 by TBA)–MeOH (960/40)	FD, 278 nm, 450 nm			LOQ = 25 ng L^{-1}	[54]
CIPRO, ENRO, FLE, FLU, LOME, MOXI, NOR, OFLO, OXO	Surface water, municipal WW, WWTP effluent, SPE	ID YMC-Pack Pro C_{18} (250 mm × 4.6 mm, 3 μm)	Gradient elution, A: 50 mM FAc, B: MeOH	FD, 278–320 nm, 365–500 nm, MS, SIM, SRM	$[M+H]^+$		LOQ_{FD} = 11–60 ng L^{-1}, LOQ_{MS} = 0.3–7.0 ng L^{-1}	[55]

Table 7
HPLC analytical methods for the determination of SAs and TMP.

Substances determined	Matrix Sample preparation	Stationary phase, analytical column	Mobile phase	Detection	Precursor ions	Internal standard	Sensitivity LOD/LOQ	Ref.
SMT, SMX, SMO, SPY, SDZ, SCP, SMR, SSM, SQX, SMP, SDT	Surface, drinking water, SPE	Alltima RP C_{18} (150 mm × 2.1 mm, 5 μm)	Gradient elution A: ACN + 1 mM FAc, B: water + 1 mM FAc	ESI$^+$, MS/MS, SRM	$[M+H]^+$		LOQ = 5–21 ng L^{-1}	[3]
SMZ, SDT, SCP, SMO	Ground water, SPE	BetaBasic-18 C_{18} (100 mm × 2.1 mm, 3 μm) (30 °C)	Gradient elution, A: ACN, B: water + 0.3% FAc	ESI$^+$, MS/MS	$[M+H]^+$	$^{13}C_6$-SMZ	LOD = 20–70 ng L^{-1}, LOQ = 70–240 ng L^{-1}	[6]
SGN, SCT, SDZ, SPY, SMR, SMZ, SDT, SSZ	Wastewaters, SPE, SPME	SPE: XTerra MS C_{18} (250 mm × 2.1 mm, 5 μm) (35 °C), SPME: Ultracarb ODS C_{18} (150 mm × 4.6 mm, 5 μm) (35 °C)	Gradient elution, A: 20 mM AmAc (0.1% FAc, pH 3.0), B: 20 mM AmAc (in 2:1 ACN/MeOH)	ESI$^+$, MS/MS, SRM	$[M+H]^+$	$^{13}C_6$-SMZ, $^{13}C_3$-caffeine	LOD_{SPE} = 2.88–9.00 ng L^{-1}, LOD_{SPME} = 9.04–55.3 μg L^{-1}	[34]
SCT, SDZ, SMX, STZ, SMR, SSX, SMT, SMZ, SMM, SMP, SCP, SDX, SDT, SM	Wastewater, SPE	Symmetry C_{18} (150 mm × 2.1 mm, 3.5 μm) (20 °C)	Gradient elution, A: 0.2% FAc in MeOH, B: 0.2% FAc in water	ESI$^+$, MS/MS, SRM	$[M+H]^+$		LOQ = 1–3 ng L^{-1}	[37]
SDZ, SCP, SMT, SQX, SDM, SMD	Surface water, SPE	LiChrospher 100 RP-C_{18} (250 mm × 4 mm, 5 μm)	Gradient elution, A: 10 mM AcAc buffer pH 3.4, B: ACN	FD, 405, 485 nm (pre-column derivatization)			LOD = 1–8 ng L^{-1}	[57]
SMP, SMO, SQX, SNT, SSM, SMX, SMR, STZ, SDZ, SMT, SDM, SDT, SPY, SSX, SCP, SM, TMP	WWTP influent and effluent, river water, SPE	Acquity UPLC BEH C_{18} (100 mm × 2.1 mm, 1.7 μm) (40 °C)	Gradient elution, A: MeOH, B: water + 0.1% FAc	ESI$^+$, MS/MS, SRM		$^{13}C_6$-SMZ	$LOD_{influent}$ = 20–200 g L^{-1} L^{-1}, $LOD_{effluent}$ = 16–120 g L^{-1} L^{-1}, $LOD_{river\ water}$ = 8–60 g L^{-1} L^{-1}	[58]
SDZ, STZ, SPY, SMZ, SMX	Surface water, wastewaters, mixed hemimicelles SPE	Diamonsil-C_{18} (250 mm × 4.6 mm, 5 μm)	Gradient elution, A: water (pH 3.4 adjusted by H_3PO_4), B: ACN–water (75:25)	UV, 260 nm			LOD = 0.15–0.35 μg L^{-1}	[63]
SAD, SDZ, STZ, SMR, SMZ, SMP, SDT, SQX	Swine wastewater, SPE	Supelcosil C_{18} (250 mm × 4.6 mm, 5 μm) (35 °C)	Gradient elution, A: 0.5% AcAc in water, B: ACN	UV, 272 nm			LOQ = 5–7.5 ng L^{-1}	[64]
SDZ, STZ, SMZ, SMX, SDT and their acetylforms	Surface water, online SPE	Nucleodur C_{18} Gravity (125 mm × 2 mm, 5 μm)	Gradient elution, A: 20 mM FAc	ESI$^+$, MS/MS, SRM	$[M+H]^+$	D_4-SDZ, D_4-STZ, $^{13}C_6$-SMZ, D_4-SMX, D_4-SDT	LOD = 1–3 ng L^{-1}	[65]

SAs themselves are not fluorescent, but they can be easily derivatized with fluorescamine to form highly fluorescent derivatives. FD with pre-column derivatization was used at λ_{exc} 405 nm and λ_{em} 485 nm, respectively [57]. HPLC–UV detection at 260 nm [63] or at 272 nm was used [64]. In some studies UV detection was only used for the optimization of chromatographic separation development and then for the determination of SAs in real samples MS detection was applied. Although, HPLC–UV or HPLC–FD have been used for the detection of SAs, these methods did not achieve good sensitivity comparing to MS detection. The sensitivity of method also depends on the purpose of the method and its application on real samples. Babić et al. [12] achieved LOD and LOQ suitable for the detection of analytes in wastewaters, where the concentrations were higher than in surface waters.

LC analyses were performed of C_{18} analytical columns for the separation in all referred methods. In all cases gradient elution was employed. Mobile phases containing MeOH or ACN were used as organic modificators. For MS detection, FAc or AmAc were added. In case of UV detection, pH of water was adjusted by phosphoric acid which can be used in UV detection.

Concerning MS/MS detection, ESI^+ mode was chosen for the ionization of SAs because of its high sensitivity. The protonated molecule $[M+H]^+$ was chosen as precursor ion for quantitation in all developed methods. The identification of individual antibiotics was mostly based on chromatographic retention time and two the most intensive transitions for each compound. SAs exhibit characteristic fragmentation in the ESI^+ mode. Protonated molecule $[M+H]^+$ is usually base peak of spectra in SIM mode. The specific fragment ions are m/z 92 $[M\text{-}RNH_2\text{-}SO_2]^+$, 108 $[M\text{-}RNH_2\text{-}SO]^+$ and 156 $[M\text{-}RNH_2]^+$. The common fragment ion, m/z 156, representing the sulfanilyl ring is used for the quantification of the majority of SAs.

In case of SAs $^{13}C_6$-sulfamethazine was used the most often as IS [6,34,58,65]. Other SAs isotope labelled compounds were employed as well, e.g. D_4-sulfadiazine, D_4-sulfathiazole, D_4-sulfamethoxazole, and D_4-sulfadimethoxine [65].

Only one work used UHPLC in tandem with MS. C_{18} column with 1.7 μm particles was employed [58].

4.7. Macrolide antibiotics

Only four methods dealed with the determination of MLs. Analytical methods for the determination of MLs employed HPLC with tandem MS (as is shown in Table 8). MLs do not have any suitable chromophore group and this causes that MLs show poor UV absorbance, indicating that specific, selective and sensitive UV detection of these compounds in environmental waters is difficult [30]. One study refers the monitoring ERY, roxithromycin (ROXI) and tylosin (TLS) by UV, but the tandem MS was used for the accurate quantitation [30]. FD of MLs is possible with their derivatization. Thus, it is clear that MS detection is the most suitable technique for this group of antibiotics from the point of view of sensitivity and specificity.

The methods for the determination of MLs employed MS/MS using specific SRM conditions with ESI^+. The precursor ion chosen for the quantitation was $[M+H]^+$ in almost all studies, except of ERY-H_2O, for which $[M+H\text{-}H_2O]^+$ was used as a precursor ion [5,9,17,30].

MLs were separated on the C_{18} analytical column. For the separation, gradient elution was utilized in all cases. ACN was used as a part of mobile phase, together with AmAc buffer of FAc to enhance ionization. MeOH was used in one study as a third part of mobile phase [9].

Isotope labelled ERY [8,18,43] was used as IS in multiresidue studies [3]. Structurally related compounds e.g., kitasamycin [17], spiramycin [9], josamycin [5,7,24] or oleandomycin [5] were also employed.

Table 8
HPLC analytical methods for the determination of MLs.

Substances determined	Matrix sample preparation	Stationary phase, analytical column	Mobile phase	Detection	Precursor ions	Internal standard	Sensitivity LOD/LOQ	Ref.
CLAR, ROXI, ERY-H_2O, SPIR, TLS	Ground water, WWTP effluent, SPE	Nucleosil 100-5 C_{18} HD (125 mm × 5 mm) (30 °C)	Gradient elution, A: 10 mM AmAc buffer (pH 6.0) + ACN (90:10), B: ACN	ESI^+, MS/MS, SIM	$[M+H]^+$	JOSA, Oleandomycin	LOQ = 2–35 ng L^{-1}, LOQ = 5–70 ng L^{-1}	[5]
ERY-H_2O, TLS	WWTP influent and effluent, SPE	Xterra MS C_{18} (50 mm × 2.1 mm, 2.5 μm) (45 °C)	Gradient elution, A: 0.1% FAc in water, B: 0.1% FAc in ACN, C: MeOH	ESI^+, MS/MS, SRM	$[M+H]^+$	SPIR	LOD = 10–60 ng L^{-1}	[9]
AZI, CLAR, ERY, ROXI, JOS	Surface water, SPE	Hypurity C_{18} (250 mm × 2.1 mm, 5 μm)	Gradient elution, A: ACN + 10 mM AmAc (pH 6.0) (10:90), B: ACN	ESI^+, MS, SIM, MS/MS, SRM	$[M+H]^+$	Kitasamycin	LOD_{MS} = 0.14–1.9 μg L^{-1}, LOQ_{MS} = 0.47–6.3 μg L^{-1}, $LOD_{MS/MS}$ = 13–140 ng L^{-1}, $LOQ_{MS/MS}$ = 43–470 ng L^{-1}	[17]
ERY-H_2O, ROXI, TLS	Surface waters, WWTP, SPE	XTerra MS C_{18} (50 mm × 2.1 mm, 2.5 μm) (45 °C)	Gradient elution, A: water + 0.1% FAc, B: ACN + 0.1% FAc	UV 215,205, 287 (resp.), ESI^+, MS/MS, SRM	$[M+H\text{-}H_2O]^+$, $[M+H]^+$, $[M+H]^+$	Simatone	LOD = 30–70 ng L^{-1}	[30]

5. Conclusions and future trends

This review summarizes recently developed analytical methodologies for the determination of different groups of antibiotics in environmental waters. A challenge is presented in the simultaneous extraction and analysis of multiple classes of compounds due to the wide range of polarities, solubilities, pK_{as} and other properties under the acidic and basic conditions.

To prevent emergence of resistant bacteria, it is necessary to monitor the concentration, fate and removal of antibiotics in environmental samples. Many studies showed the incomplete elimination during wastewater treatment processes, thus the next task is to improve the treatment process.

Detailed knowledge of the behaviour of antimicrobials in wastewater treatment and the aquatic environment will help to achieve a reliable basis of environmental risk assessment (e.g., by providing measured environmental concentrations, MECs). MECs can be used in environmental risk assessment studies since they provide accurate indications of actual concentration present in environmental systems. Investigations of the occurrence and fate of antimicrobial agents in various wastewater treatment steps can be exploited in order to evaluate wastewater treatment technologies with the respect to elimination of specific contaminants. Reducing the release of residual antibiotics into the aquatic environment would presumably decrease any potential environmental risks. During monitoring receiving surface waters as well as wastewater treatment plants, location of particular concern can be identified and mitigated specifically.

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5.6. Supplement II

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Determination of fluoroquinolone antibiotics in hospital and municipal wastewaters in Coimbra by liquid chromatography with a monolithic column and fluorescence detection.

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ORIGINAL PAPER

Determination of fluoroquinolone antibiotics in hospital and municipal wastewaters in Coimbra by liquid chromatography with a monolithic column and fluorescence detection

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Abstract The main goal of this work was determination of residues of the antibiotics ofloxacin (OFLO), norfloxacin (NOR), ciprofloxacin (CIPRO), and enrofloxacin (ENRO) in wastewater samples. The samples, after acidification to pH 4.5 and addition of EDTA, were extracted on an anion-exchange cartridge in tandem with an Oasis HLB cartridge. The LC–FD method, developed in previous studies, was based on application of a monolithic C_{18} column. The limit of quantification (LOQ) of the method was 250 ng L^{-1} for OFLO, 25 ng L^{-1} for NOR and CIPRO, and 50 ng L^{-1} for ENRO. Mean recovery ranged between 75 and 121% for OFLO, NOR, CIPRO, and ENRO. A total of 14 wastewater samples were analyzed; these were collected from four hospitals and from influent and effluent from a wastewater-treatment plant in Coimbra, Portugal, during spring and autumn. CIPRO was present in all the samples, NOR was detected second most often, followed by OFLO. ENRO was found at concentrations under the LOQ in five hospital samples, and the highest level was found in influent from the WWTP.

Keywords Fluoroquinolones · LC · Monolithic column · Pharmaceuticals · Hospital and municipal wastewater

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Introduction

Pharmaceuticals are emerging environmental contaminants that have caused increased concern in recent years, because studies have indicated the presence of antibiotics in hospital and municipal wastewaters [1] that may enter aquatic systems. Antibiotics residues have been found in a wide range of environmental samples, including surface water, ground water, and drinking water [5]. The selection and development of antibiotic-resistant bacteria is one of the greatest concerns regarding the use of antibiotics [6].

Despite the large quantities used, published data on the amounts and patterns of use of antibiotics are scarce. Generally, fluoroquinolones (FQ) are prescribed in human medicine between 300–600 mg per day for therapeutic treatment of the patient. They are almost all excreted as unchanged compounds in the urine and are, consequently, discharged into hospital sewage or municipal wastewater [7]. Hospital wastewater is one of the main sources of contamination. Antibiotic concentrations calculated and measured in hospital effluents are of the same order of magnitude as minimum inhibitory concentrations for susceptible pathogenic bacteria [8]. Depending on treatment of the sewage water, different concentrations of the active compounds are found after treatment and are transported into aquatic systems [5, 9, 10, 11]. In recent years, several initiatives have been launched to establish or strengthen surveillance systems, both in EC member states and at an international level, to monitor the presence of antibiotic residues in environmental waters; these have revealed levels of antibiotic residues in this type of sample.

Several analytical methods have been developed to extract, concentrate, and quantify FQ residues in environmental water samples. There are reports of use of solid-phase extraction (SPE), for extraction and concentration of FQ, and analysis by

liquid chromatography (LC) with fluorescence detection (FD) [11, 12] ultraviolet detection (UV) [13], and mass spectrometric (MS) [14] or tandem mass spectrometric (MS–MS) detection [15].

This paper describes the development and validation of analytical methodology for specific and sensitive determination of the FQ antibiotics ofloxacin (OFLO), norfloxacin (NOR), ciprofloxacin (CIPRO), and enrofloxacin (ENRO), in hospital and municipal wastewaters. The methodology is based on tandem SPE (SAX and Oasis HLB cartridges) and sensitive LC–FD analysis, using a Chromolith RP-18e monolithic column [16]. Our work focussed on estimation of the presence of the compounds in four hospital wastewaters, because these are an important route of entry of human-use antibiotics into the environment, and into influents and effluents of a municipal wastewater treatment plant (WWTP) in Coimbra, Portugal. Because WWTPs are considered to be point sources of antibiotic contamination of surface and ground waters, it is very important to predict the concentration of these antibiotics and to evaluate the efficiency of the treatment. Some preliminary results from field measurements are reported.

Experimental

Reagents

Standards of OFLO, NOR, CIPRO and ENRO were purchased from Sigma–Aldrich (Steinheim, Germany). These FQ were >98% purity. LC-grade methanol was supplied by Carlo Erba (Milan, Italy), phosphoric acid RPE-ACS by Carlo Erba (Milan, Italy), sulfuric acid 95–97% Reagente "Baker Analyzed" by Baker (Deventer, The Netherlands), tetrabutylammonium hydroxide (TBA) by Sigma-Aldrich, and EDTA by Merck (Germany). Water was HPLC grade. The cartridges used for SPE were Oasis HLB 6 cc/200 mg (Waters, Milford, MA, USA) and AccuBOND II SAX Cartridges 6 mL/500 mg (Agilent Technologies, Santa Clara, USA).

Apparatus and chromatographic conditions

The LC method described here was performed with one pump (model 307, Gilson Medical Electronics, France), a Model 7125 injector (Rheodyne, Cotati, California, USA), and a Perkin–Elmer (Beaconsfield, UK) model LS45 spectrofluorimeter operated at an excitation wavelength of 278 nm and an emission wavelength of 450 nm. The spectral bandwidth was 10 nm for both excitation and emission. The results were recorded by FL WinLab Software and a 3390A integrator (Perkin–Elmer). The four FQ were eluted isocratically 0.025 mol L^{-1} phosphoric acid solution (pH adjusted to 3.0 with TBA–methanol–acetonitrile 920:70:10 (*v*/*v*) as mobile phase. Analysis was performed on a monolithic column (Chromolith Performance RP-18e; 100×4.6 mm) at a flow rate of 1.2 mL min^{-1} and at room temperature.

Sample collection and preparation

A total of fourteen 24-h composite wastewater samples from the four hospitals situated in Coimbra and from the influents and effluents of the local wastewater-treatment plant, were collected during the spring and autumn of 2007. Wastewater samples were collected in amber glass bottles and kept in a cooler with ice during transportation. After delivery to the laboratory, samples were filtered through 0.45-μm glass fibre filters, to remove suspended matter, and stored in the dark at 4° C until extraction, which occurred within two days.

Standard fluoroquinolone solutions

Individual stock standard solutions (1 mg mL^{-1}) were prepared in 0.005 mol L^{-1} sulfuric acid. The working standard solutions were a mixture of the four compounds prepared by appropriate dilution of the stock solutions with 0.005 mol L^{-1} sulfuric acid.

Extraction and clean-up

Prior to extraction, wastewater samples were filtered through 0.2 μm membrane filters. After acidification to pH 4.5 and addition of EDTA, they were extracted through an anion-exchange cartridge in tandem with an Oasis HLB cartridge. The cartridges were previously pre-conditioned with 2 mL methanol followed by 2 mL of citric acid (pH 4.0). The samples were percolated at a flow rate of approximately 3 mL min^{-1}. After sample percolation, the cartridges were dried for a while and then the SAX cartridge was removed and the washing step was performed with the Oasis HLB cartridge only. The cartridge was washed with 2 mL citric acid (pH 4.0) and 20 mL Milli-Q water at pH 4.2. Afterwards, they were vacuum dried for 15 min. Subsequently, the analytes were eluted with 4 mL methanol.

Clean-up efficiencies were studied to monitor the effect of pH on retention of the FQ, the effects of the solvents and pH used in the preconditioning and washing steps, and the effects of the nature and volume of the eluent solvents.

Before injection into the chromatographic system, the methanolic eluate was concentrated to dryness under a gentle stream of nitrogen, redissolved in mobile phase, and filtered through a 0.45-μm membrane filter.

Results and discussion

Optimization of HPLC conditions

Our previous experiments showed that with Chromolith column the best result in terms of resolution and selectivity were achieved at pH 3.0 in the presence of TBA [16], which is in accordance with the results of Forlay–Frick and Fekete [17]. A Chromolith Performance RP-18e column (100×4.6 mm) was used with a 10-mm precolumn, at room temperature; separation was accomplished in 12 min with an isocratic mobile phase—a 920:70:10 (*v*/*v*) mixture of phosphoric acid adjusted to pH 3.0 with tetrabutylamonium hydroxide (TBA), methanol, and acetonitrile, at a flow rate of 1.2 mL min^{-1}.

On the basis of three parallel determinations, on three days, the mean retention times and relative standard deviation (RSD, %) for OFLO, NOR, CIPRO, and ENRO were 4.47, 6.50, 7.59, and 10.70 min, and 0.52%, 0.39%, 0.40%, and 1.17%, respectively.

Excitation–emission scans were performed to establish optimum excitation and emission wavelengths. The maximum wavelengths obtained for the different fluoroquinolones were: for OFLO λ_{ex} 277 nm, λ_{em} 490 nm, for NOR λ_{ex} 280 nm, λ_{em} 447 nm, for CIPRO λ_{ex} 279 nm, λ_{em} 445 nm, and for ENRO λ_{ex} 277 nm, λ_{em} 446 nm. Chromatographic detection was therefore performed at excitation and emission wavelengths of 278 nm and 450 nm, respectively.

A previous interference study showed that the solvent used to prepare standard solutions of OFLO, NOR, CIPRO, and ENRO has no effect in fluorescence intensity [16].

Optimization of extraction and clean-up

Acidification of the sample with a weak acid (acetic acid) at pH 4.5 was selected because the FQ, with a piperazinyl moiety, are fully protonated at low pH. EDTA was added as chelating reagent, because the FQ form stable complexes with Ca (II), Mg (II), and Al (III) by ion–dipole interaction with the 4-keto oxygen and the ionized 3-carboxylic acid groups [18].

The cleanup efficiencies were studied to monitor the effects of the type of cleanup cartridge, the solvents used in the preconditioning and washing steps, the solvents used for elution, and the volumes for eluting FQ from the cartridges. When Oasis HLB cartridges (poly(divinylbenzene–co-*N*-pyrrolidone) were used in the Pena et al. [16] method for determination of FQ residues in surface waters some interferences were observed at the retention times of the FQ. Under our experimental conditions, this cleanup was not efficient in removing the interferences from this type of water sample.

As reported by other authors [12–14, 19], our findings showed that a more effective cleanup procedure that can separate FQ from impurities present in wastewater samples is essential. Turiel et al. [20] also observed that a single cleanup through a C_{18} cartridge was not always sufficient for analysis of FQ residues in this type of matrix. Recently, tandem SPE methods using a strong anion-exchange (SAX) cartridge and an HBL cartridge were studied for soil and pig slurry samples [21]. Organic matter reduces extraction efficiency and can interfere with detection [22]. The anion-exchange cartridges reduce matrix interferences by adsorbing negatively charged humic material and other highly negatively charged natural organic matter from the wastewater samples, thus preventing contamination, blockage, and overloading of the HLB sorbent. We applied the cleanup described by Jacobsen et al. [23] for wastewater extracts—SPE on SAX (strong anion-exchange) cartridges in tandem with Oasis HLB cartridges.

At pH 4.5, FQ are in the cationic form, because of deprotonation of the carboxylic acid group and protonation of the piperazinyl amino group [22], and therefore are not retained on the SAX cartridge, while the polymer Oasis HLB adsorbent with an equal ratio of hydrophilic *N*-vinylpyrrolidone and lipophilic divinylbenzene retained the FQ. They have both an acidic and basic functional groups that can interact with either hydrophilic or lipophilic portion, of the Oasis HLB adsorbent [24].

Because of the acid–base properties of the FQ, SPE of the analytes is expected to be strongly dependent on pH. During this optimization stage, different buffers for cartridge conditioning and different sample pH were evaluated. Samples were evaluated at pH 4.0, 4.5, 5.0, 5.5, and 6.0 and cartridges were conditioned with 4.38 mmol L^{-1} phosphoric acid, citric acid buffer at pH 4.7 and 4.0, and acetate buffer at pH 5.5. In the Renew method [22] the cartridges were conditioned with methanol and 4.38 mmol L^{-1} phosphoric acid (pH 3.3), and recovery ranged from 67 to 120%. Under our experimental conditions, better results were obtained with the sample at pH 4.0.

When a second assay was performed with acetate buffer at pH 5.5 the overall efficiency of recovery of FQ was: higher than 100% at pH 4.0 and from 59 to 85% and from 55 to 84% at pH 5.0 and 5.5, respectively. According to Jacobsen et al. [23] the use of 0.04 mol L^{-1} citric acid buffer (pH 4.7) results in recovery higher than 100% at pH 4.0 and 5.0, and recovery between 60 and 86% at pH 6.0.

Finally, citric acid buffer at pH 4.0 was evaluated. The washing step was performed with citric acid buffer at pH 4.0 and water (without pH adjustment). Recovery values for pH 4.0, 5.0 and 5.5 varied from 82 to 148%. The best recoveries, ranging from 90 to 130%, were achieved for all analytes at pH 4.5. In order to improve the washing step, citric acid buffer at pH 4.0 and water at pH 4.2 were used.

The recoveries achieved for all analytes were in the range 96–114%. Because this procedure gave the best recoveries for the FQ, it was selected and used in the analysis of wastewater samples.

After sample percolation, the Oasis cartridge was washed with 2 mL 0.04 mol L^{-1} citric acid buffer (pH 4.0) and 20 mL H_2O at pH 4.2, and dried under vacuum for 15 min. FQ were eluted from the Oasis HLB cartridge with 4 mL methanol.

To verify the absence of interfering substances around the retention times of FQ different types of real sample were analyzed. No interference was detected in any of the samples analyzed and FQ recovery ranged between 75 and 120%.

Method validation

In validation of the analytical method, the criteria sensitivity, linearity, recovery, and precision, and evaluation of matrix effects were considered.

Linearity

The calibration curves were prepared using linear regression analysis and over the established in the range gave good fits. The mean regression coefficients (r^2) were 0.9985 for OFLO, 0.9994 for NOR, 0.9999 for CIPRO, and 0.9975 for ENRO.

Stability studies

The stability of standard solutions and of sample extracts was evaluated. Stock standard solutions were stored at −20° C and analysed during a one-month period, and working standard solutions were stored at 4° C and analysed during a one-week period. The stability of FQ during sample storage at −20° C was tested for one week; after three days, degradation of FQ working standard solutions was observed.

Limit of detection, limit of quantification, and accuracy

The limit of detection (LOD) was 85 ng L^{-1} for OFLO, 8.5 ng L^{-1} for NOR and CIPRO, and 17 ng L^{-1} for ENRO. The limit of quantification (LOQ), calculated as the lowest concentration that provides repeatabilities better than 20%, was 250 ng L^{-1} for OFLO, 25 ng L^{-1} for NOR and CIPRO and 50 ng L^{-1} for ENRO.

In order to verify the absence of potential interfering substances around the retention time of FQ, and to assess the specificity of the method, water blank samples (n = 4) were analyzed. No interferences were observed in the regions of interest where the FQ were eluted. These results demonstrate that real sample matrices had no effect on the performance of the proposed method, which is, therefore, suitable for analysis of trace levels of FQ in wastewaters.

The accuracy of the method was studied by spiking water samples at three fortification levels (100 ng L^{-1} for NOR and CIPRO, 200 ng L^{-1} for ENRO, and 1,000 ng L^{-1} for OFLO; 200 ng L^{-1} for NOR and CIPRO, 400 ng L^{-1} for ENRO, and 2,000 ng L^{-1} for OFLO; and 500 ng L^{-1} for NOR and CIPRO, 1,000 ng L^{-1} for ENRO, and 5,000 ng L^{-1} for OFLO). Mean recoveries ranged between 75 and 121% for OFLO, NOR, CIPRO, and ENRO.

Within-day accuracy and precision data were determined by analysing, on the same day, three replicates of a spiked sample at three fortification levels, and one blank (to check for interferences). The between-day accuracy and precision were also determined by extracting batches of three fortification levels and analysing them on three consecutive days. For the three fortification levels, the relative standard deviation for all fortification levels on each day for each analyte was less than 10%, showing good method precision.

Application of method

A total of 14 wastewaters samples collected during spring and autumn from four hospitals and from influent and effluent water from a wastewater-treatment plant (WWTP) were analysed. The measured concentrations of the FQ in the samples studied are summarized in Tables 1 and 2. Typical chromatograms are shown in Fig. 1.

Concerning the frequency of detection, CIPRO was found, in all the samples analysed (100%), NOR in 79%, and OFLO in 50%. It was not surprising that the highest concentration was for CIPRO and OFLO, which are assumed to be human-influence antibiotics. These results underscore the importance of development of this type of monitoring study. As mentioned above, high levels of CIPRO and OFLO, ranging from 100.8 to 10,962.5 ng L^{-1} and 353.3 to 10,675.5 ng L^{-1}, respectively, were found. NOR was present at concentrations between 29.6 to 455.0 ng L^{-1}. ENRO was found in WWTP influent, with a frequency of 29%, ranging between 53.7 and 447.0 ng L^{-1}, and was detected under the LOQ in five hospital wastewater samples.

In Germany, Hartmann et al. [2] also reported the presence of FQ in hospital wastewaters, at concentrations varying from 3 to 87 μg L^{-1}. In Sweden [7] CIPRO was detected in the range from 3.6 to 101.0 μg L^{-1} in hospital sewage water and in a study from the USA [5], OFLO and CIPRO were found at concentrations of 4,900–34,500 ng L^{-1} and 850–2,000 ng L^{-1}, respectively.

Differences were observed in wastewaters from the different hospitals. In hospitals 1 and 3, OFLO, NOR, and CIPRO were detected in both seasons. NOR and CIPRO were also found in branch 1 of hospital 4 during spring and autumn.

Table 1 Concentrations (ng L^{-1}) of FQ in wastewater samples collected during spring

Sample	OFLO	NOR	CIPRO	ENRO
Hospital 1	2,289.0	228.9	2,893.0	n.q.[a]
Hospital 2	3,008.3	–	1,554.5	–
Hospital 3	353.3	134.5	1,926.9	n.q.
Hospital 4				
Branch 1	–	29.7	10,962.5	–
Branch 2	10,675.5	–	3,388.7	–
Branch 3	–	n.q.	127.0	n.q.
Wastewater-treatment plant				
Influent	–	191.2	667.1	447.1
Effluent	–	29.6	309.2	211.5

[a] Not quantified (<LOQ)

A seasonal influence on the frequency of detection of FQ in hospital wastewaters was observed, and was evident especially for OFLO, NOR, and CIPRO. Results obtained from hospitals 1 and 2, in both seasons, show comparable values of CIPRO and NOR, with the exception of OFLO. In water from hospital 2, the presence of NOR was not observed in the samples collected during spring, and OFLO was present at a higher concentration during autumn in water from hospital 1.

For hospital 3 the CIPRO level was three times higher during spring, and OFLO was approximately five times higher during the autumn.

Concerning samples from hospital 4, although it was impossible to collect samples in the three different branches in both seasons, we can state, in a general way, that for samples collected from branch 1 during spring and autumn higher concentrations of OFLO and CIPRO were found in spring.

Influent and effluent samples from WWTPs, contained NOR, CIPRO, and ENRO at lower levels than in hospital wastewater samples. These results may be explained by a dilution effect with the municipal wastewaters, or degradation processes in the aquatic environment.

Regarding the influence of season in this type of sample, we can assume that the FQ studied were detected at lower levels during the autumn season, except for NOR.

Table 2 Concentrations (ng L^{-1}) of FQ in wastewater samples collected during autumn

Sample	OFLO	NOR	CIPRO	ENRO
Hospital 1	9,451.9	334.0	2,927.2	–
Hospital 2	3,111.6	197.3	1,007.3	n.q.[a]
Hospital 3	1,585.5	88.5	619.9	–
Hospital 4 branch 1	–	301.6	726.3	n.q.
Wastewater-treatment plant				
Influent	–	455.0	418.8	121.8
Effluent	–	35.0	100.8	53.7

[a] Not quantified (<LOQ)

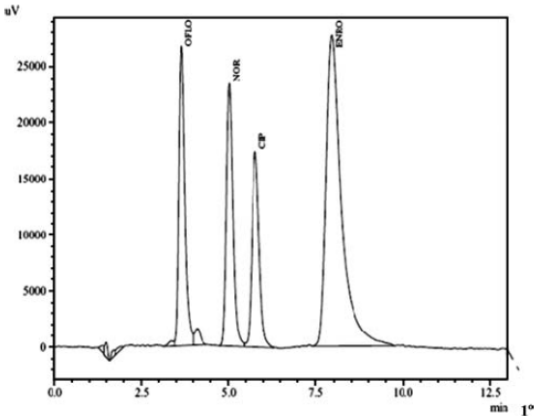

Fig. 1 Liquid chromatograms obtained from a standard solution and hospital wastewater

In the samples from the WWTPs, concentrations before and after treatment during spring were 191.2 and 29.6 ng L^{-1}, 667.1 and 309.1 ng L^{-1}, and 447.1 and 211.5 ng L^{-1}, for NOR, CIPRO, and ENRO, respectively. For samples collected during autumn, the values were, respectively, 455.0 and 35.0 ng L^{-1}, 418.8 and 100.8 ng L^{-1}, and 121.8 and 53.7 ng L^{-1}.

In the scientific literature FQ have been reported in effluents from sewage treatment plants in European countries, such as France (330–510 ng L^{-1}), Italy (290–580 ng L^{-1}), Greece (460 ng L^{-1}) [11], Switzerland (36–106 ng L^{-1}) [9], (249–405 ng L^{-1} primary effluent, 45–120 ng L^{-1} tertiary effluent) [12], and also in USA (19–45 ng L^{-1}) [11] (110–470 ng L^{-1}) [15] and Canada (34–179 ng L^{-1}) [19], (112–506 ng L^{-1}) [25]. OFLO was detected in the effluents of WWTPs in several European countries [11], although it was not detected in our study. These different results might be due to variations in the use of FQ among countries. ENRO, used only in veterinary medicine, was detected in our study in municipal wastewaters, probably because of agricultural sources, such as manure dispersion and animal excretion on to soils.

Because CIPRO, ENRO, and OFLO have high K_{dsolid} values, ranging between 496 and 61,000 L kg^{-1} [26], and because to their lipophilicity and tendency to form stable complexes with divalent and trivalent metal ions, they move rapidly from the water compartment into solids. Therefore, sorption to sewage sludge through hydrophobic interactions has been suggested as the main removal pathway for FQ during secondary wastewater treatment [4, 27] because of their immobility as a result of the high sorption coefficients for solids.

The percentage of reduction in dissolved FQ concentrations after treatment was: 85 and 92% for NOR, 54 and 76% for CIPRO, and 53 and 56% for ENRO, in spring and autumn, respectively. These results are in accordance with other

reported studies [4]. The extent of reduction observed in our study was fairly high in autumn. However, it should be noted, that the weather was unusually warm when samples were collected during this season. This is in correlation with a study from Switzerland [9], where FQ were significantly reduced in WWTP process during the summer period than in winter.

The concentration of FQ in the final effluent from the WWTPs depends on the treatment process used. In a field study at a full-scale municipal wastewater-treatment plant in Switzerland, Golet et al. [27] determined a 49–61% reduction in dissolved CIPRO and NOR concentrations during biological treatment, 28–35% during mechanical treatment, and about 3–4% was removed in the flocculation filtration step. They observed the combined removal during the wastewater treatment process of 88% for CIPRO and 92% for NOR. In another study of Golet et al. [12] they observed a rate of elimination during wastewater treatment between 70 and 80% after advanced treatment with contact filtration. Thus the main removal results from biological treatment.

The efficiency of antibiotics removal in WWTPs based an activated sludge process and biological nutrient removal has been evaluated [5]. Only OFLO was found in influent and effluent from WWTPs in the USA and removal efficiency was 77%. FQ are known to readily sorb to sewage sludge, which may explain their higher removal rate. Lee et al. [19] observed reduction of OFLO, NOR, and CIPRO by more than 40% during the treatment process in WWTPs in Canada.

These results are in concordance with those from Switzerland where they evaluated the efficiency of the treatment process in WWTP. The reduction in concentrations if NOR and CIPRO after treatment was reported to be in the range 79–87% [9]; another study reported a reduction of 70–80% for CIPRO [13]. In Sweden [15] the efficiency reported was 87% for NOR, 86% for CIPRO, and 87% for ENRO.

CIPRO has been detected in WWTP effluents and its concentration differs according to the efficiency of treatment (22.2–100%), which depends on the secondary treatment process used. For use of an oxidation ditch and activated sludge reduction of CIPRO was 22.2 and 71.4%, respectively [10].

In a study by Renew et al. [22] they compared the efficiency of two WWTPs. Both treatment plants utilize primary and secondary (activated sludge) treatment. After this secondary treatment, one WWTP utilizes chlorination whereas the second WWTP utilizes UV disinfection. From their results they concluded levels of antibiotics were comparable after secondary treatment whereas the concentration of antibiotics differed substantially in tertiary effluents. WWTP using chlorination led to lower quantities of the antibiotics studied than the WWTP with UV disinfection.

Advanced wastewater treatment techniques, for example reverse osmosis, activated carbon, and ozonation have been shown to significantly reduce or eliminate antibiotics from wastewater effluent [5].

Therefore, more studies are necessary in order to evaluate the efficiency of the different types of treatment at local WWTPs and to avoid the entrance of antibiotic residues into the aquatic environment.

Conclusions

A method including tandem SPE (an anion-exchange cartridge and an Oasis HLB cartridge) and LC–FD analysis using a monolithic column is proposed for simultaneous determination of OFLO, NOR, CIPRO, and ENRO in hospital and municipal wastewaters.

The analytical method was found to fulfill validation requirements of linearity, accuracy, precision, and selectivity for FQ determination in wastewater samples, and may be used for routine analysis because LC–FD is an inexpensive analytical technique compared with LC–MS. This analysis can be a useful tool to determine the amount of FQ discharged from WWTPs into the aquatic environment and evaluate the effect of the treatment in their elimination. Our results show reduction of the concentrations of the antibiotics in the range 53 to 92%. This correlates with studies from other countries and showed that elimination during treatment process in not complete and it is necessary to improve treatment processes and to prevent entrance of antibiotics into the aquatic environment and their persistence there.

The relatively high concentrations of antibiotics found in this work, ranging from 100.8 to 10,962.5 ng L^{-1} for CIPRO, from 353.3 to 10,675.5 ng L^{-1} for OFLO, and from 29.6 to 455.0 ng L^{-1} for NOR, support the statement that hospitals are primary and very important contributors of antibiotics to municipal wastewaters, and underscore the importance of development of local and nationwide surveys of antibiotics in Portugal.

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5.7. Supplement III

Marcela Seifrtová, Jana Aufartová, Jitka Vytlačilová, Angelina Pena, Petr Solich, Lucie Nováková

Determination of fluoroquinolone antibiotics in wastewater using ultra high-performance liquid chromatography with mass spectrometry and fluorescence detection.

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Research Article

Determination of fluoroquinolone antibiotics in wastewater using ultra high-performance liquid chromatography with mass spectrometry and fluorescence detection

A new ultra HPLC (UHPLC) method using both MS and fluorescence detection (FD) was developed for the determination of five fluoroquinolones in wastewaters. Systematic method development approach was compared with a conventional one. During the systematic approach, a possibility of automatic switching among four independent analytical columns of different chemistries has been used. Acidic as well as basic pH using ACN and methanol as organic modifiers was tested. The best separation of fluoroquinolones was obtained on phenyl analytical column at pH 10.5, which is a completely novel approach for separation of fluoroquinolones. Further, a new SPE procedure was developed for the sample preparation using basic pH as well. The sensitivity and selectivity of FD and MS detection were compared. FD at basic pH 10.5 demonstrated lower sensitivity than at acidic pH, which is conventionally performed. At basic pH, UHPLC-MS/MS was found about two orders of magnitude more sensitive than FD. Both methods were validated and subsequently UHPLC-FD method was used for the evaluation of stability of fluoroquinolones. UHPLC-MS/MS method was used for the analysis of wastewater samples. Norfloxacin and ciprofloxacin were detected in samples of influent and effluent from wastewater treatment plant. Ofloxacin was detected only in influent from wastewater treatment plant.

Keywords: Fluoroquinolones / Systematic method development / Ultra HPLC / Wastewaters
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1 Introduction

Consumption of antibiotics grows up significantly every year as they are a group of pharmaceuticals widely used in treatment of various bacterial infections. Fluoroquinolones (FQs) are an important group of broad-spectrum synthetic antibacterial agents used for the treatment of gram-negative bacterial infections in both human and veterinary medicine [1]. FQs, as other pharmaceuticals, commonly enter into the environment mainly *via* urine and faeces, hospital wastewater and from veterinary use [2]. Several investigations have shown some evidence that pharmaceuticals are often not eliminated during wastewater treatment process in wastewater treatment plant (WWTP) [3]. The mechanism of their elimination is sorption to sewage sludge [2] and it leads to removal rate approximately between 80 and 90% [4, 5]. Another very important source of contamination with large variety of pharmaceuticals is hospital wastewater [5]. Antibiotics occur there at higher concentrations (μg/L) [5] than in municipal wastewaters (ng/L) [3, 6]. There are two reasons for this fact: their high usage in hospitals and high dilution of municipal wastewaters. FQs can persist in the environment as they have been shown to bind to solid particles [7]. Consequently, FQs may contaminate agricultural fields, disturb natural balance and accumulate in crops and vegetables [8]. Even very small amounts of antibiotics in everyday food may generate the strains of resistant bacteria in human and animal bodies, induce allergy and affect the liver [9]. The main risk for the public health is nevertheless the ability of FQs to induce the development of bacterial resistance.

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Abbreviations: AmAc, ammonium acetate; **AmF**, ammonium formate; **AQC**, Acquity; **CIPRO**, ciprofloxacin; **DANO**, danofloxacin; **DIFL**, difloxacin; **ENO**, enoxacin; **ENRO**, enrofloxacin; **FAc**, formic acid; **FD**, fluorescence detection; **FLU**, flumequine; **FQ**, fluoroquinolone; **LOME**, lomefloxacin; **MeOH**, methanol; **NAL**, nalidixic acid; **NOR**, norfloxacin; **OFLO**, ofloxacin; **OXO**, oxolinic acid; **PEFLO**, pefloxacin; **SARA**, sarafloxacin; **SRM**, selected reaction monitoring; **SST**, system suitability test; **UHPLC**, ultra HPLC; **WWTP**, wastewater treatment plant

Despite lots of studies with positive detection of antibiotics and other pharmaceuticals in soils and environmental waters and despite of their negative effects on human health, there is no defined limit value for the occurrence of these pollutants in soils or natural waters. European Union requires maximum residue limits for the sum of enrofloxacin (ENRO) and its metabolite ciprofloxacin (CIPRO) for all food producing animals. For example, in cattle the maximum residue limits are 300 μg/kg in liver and 200 μg/kg in kidney [10].

FQs are characterized by fluorine atom at position 6 of the quinolone naphthyridine or benzoxazine ring (Fig. 1) [11]. They have two relevant ionizable functional groups, the 3-carboxyl group and *N*-4 of the piperazine substituent. Therefore, FQs have two pK_a values and their acid–base behavior will be significantly affected by physicochemical properties of the solvent [2]. Reported values of pK_a for carboxylic group range from 5.7 to 6.3, whereas those for protonated amino group are higher (7.6–8.3). The intermediate form of FQs is a zwitterion [12].

Several methods for the determination of FQs in environmental matrices including surface waters, wastewaters, WWTPs effluents and soils have been recently published (Table 1). LC with fluorescence detection (FD) or MS detection was mostly employed in environmental applications. Only in one study, capillary electrophoresis was used for the separation of FQs in biological and environmental samples [13]. FQs are naturally highly fluorescent compounds and thus FD is suitable for their detection. However, in comparison with MS, FD was found to be less sensitive and some impurities or other fluorescent compounds could interfere with their sensitive and specific detection [14]. This problem may occur especially in complex environmental matrices such as wastewaters where many other interfering compounds are typically present. Therefore, a coupling of LC-MS or LC-MS/MS is a method of choice for selective determination of FQs and also other pharmaceuticals in wastewaters.

SPE is the crucial step in environmental analysis as it is necessary to preconcentrate analytes because of very low concentrations typically found in environmental waters and due to the complexity of this matrix. Sample cleanup influences further analysis especially when MS detection is employed as ESI source is highly susceptible to matrix interference. SPE is the most widely used procedure for the sample pretreatment of FQs in environmental matrices (Table 1). Only one study used solid-phase microextraction [15]. The most widely used SPE cartridges were polymeric Oasis HLB cartridges because their lipophilic divinylbenzene and the hydrophilic *N*-vinylpyrrolidone composition allows the extraction of polar and nonpolar analytes and the cartridges might be used within a wide pH range.

For the separation of FQs, C18 or C8 analytical columns were used (Table 1). In two studies, monolithic columns were employed [4, 16], whereas other methods applied conventional particulate columns. In all cases, the separation was done with mobile phase of acidic pH below the first pK_a value. Ferdig *et al.* [17] reported the problem of separation of norfloxacin (NOR) and CIPRO. The separation of these two antibiotics is challenging as they differ only by a small side group (ethyl and cyclopropyl (Fig. 1)). Toussaint *et al.* [18] compared C8 and C18 analytical columns and C8 stationary phase was chosen in order to reduce the retention time of acidic FQs and further to improve the symmetry of the chromatographic peaks. Only one study referred using phenyl analytical column for the separation of FQs together with tetracycline antibiotics by HPLC-FD [19]. The analyses were performed at pH 6.5 on the basis of already published article. However, the analysis took more than 25 min and some analytes were not baseline separated although it was sufficient for successful quantitation. Low sensitivity of FD at this pH was another drawback of this method. At this pH, FQs do not demonstrate sufficient fluorescence compared to the pH range of 3–4 [19] which might be the reason why almost all authors choose lower pH for FD. However, the selection of pH 6.5 was made as a compromise to ensure sufficient fluorescence for both classes of antibiotics studied.

Some of the developed HPLC methods [3, 12, 17] were highly time consuming (up to 40 min) and high amounts of organic solvents were used which is contrary to current trends in analytical chemistry that are miniaturizing equipment, lowering solvent consumption and of course decreasing the time of analysis. Ultra HPLC (UHPLC) allowed high separation efficiency and resolution, high sensitivity and much lower solvent consumption using sub-2-μm particles. This led to fast and high-resolution analysis compared to HPLC with conventional particle size sorbents. There was only one study employing UHPLC with MS

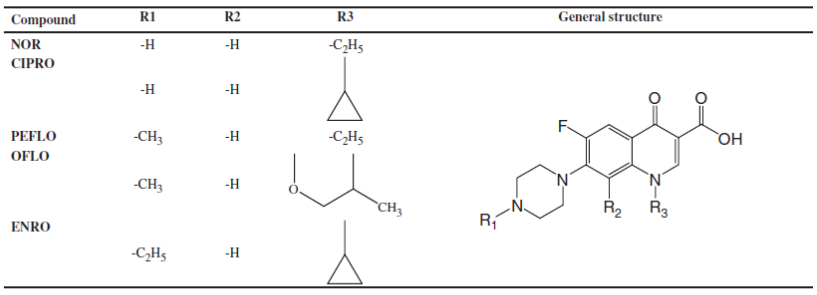

Figure 1. Chemical structures of selected fluoroquinolones

Table 1. An overview of methods for the determination of FQs in environmental waters[a)]

Substances determined	Matrix sample preparation	Stationary phase analytical column	Mobile phase	Detection	Analysis time (min)	Validation data	Ref.
9FQs: CIPRO, DIFL, ENRO, LOME, NOR, OFLO, PIP, SARA, TOS	WWTP effluents, surface water (river and lake water) SPE (150–500 mL, pH 3)	YMC ODS-AQ S-3 (50 × 4.0 mm) Discovery RP-Amide C16 (50 × 4.0 mm)	A: water (pH 3.0)+ACN (98:2) B: ACN 5:95, gradient	ESI-MS $[M+H]^+$ FD Exc: 278 nm Em: 445–500 nm	35	r^2>0.9992 LODMS = 8.6–49 ng/L	[3]
4FQs: OFLO, NOR, CIPRO, ENRO	Wastewater SPE (pH 4.5)	Chromolith Performance RP-18e (100 × 4.6 mm)	0.025 M H3PO4 (pH 3.0 by TBA): MeOH/ACN 920:70:10, isocratic	FD Exc: 278 nm Em: 450 nm	12	r^2>0.997 LOD = 8.5–85 ng/L LOQ = 25–250 ng/L	[4]
20FQs: PIP, FLE, OFLO, PEFLO, ENO, NOR, CIPRO, DANO, ENRO, LOME, DIFL, SARA, GATI, SPAR, MOXI, CINO, OXO, NAL, FLU, PIRO IS = NOR-d_5	Wastewaters, river water SPE (200–800 mL, pH 3)	ACQ UPLC BEH C18 (100 × 2.1 mm, 1.7 μm)	MeOH/0.1% FAc 10:90, gradient	ESI-MS/MS $[M+H]^+$	12	r^2>0.996 LOD = 0.6–50 ng/L	[6]
10FQs: CIPRO, DANO, DIFL, ENRO, FLU, MARBO, NAL, NOR, OXO, SARA	Surface water (seawater, well water) SPE (250–500 mL, pH 5.5)	Inertsil C8 (250 × 4.6 mm, 5 μm)	A: 10 mM oxalic acid buffer (pH 4)+ACN (89:11, v/v) B: ACN, gradient	FD Exc: 248–297 nm Em: 361–507 nm	35	LOD = 0.05–1 μg/L	[12]
3FQs: OFLO, NOR, CIPRO IS – CIPRO-$^{13}C_3{}^{15}N$	WWTP effluents SPE (250 mL, pH 3)	Zorbax SB-C8 (150 × 2.1 mm, 3.5 μm)	ACN/MeOH/FAc/water 6:12:0.5:81.5, isocratic	FD Exc: 278 Em: 450 nm ESI-MS ESI-MS/MS $[M+H]^+$	20	r^2>0.999 RSD <6% LOQ = 2–10 ng/L	[14]
5FQs: ENO, OFLO, CIPRO, NOR, LOME	Surface water, wastewater SPME	CAPCELL PAK C8 (100 × 2.1 mm, 5 μm)	5 mM AmF (pH 3)/ACN 85:15, isocratic	ESI-MS/MS $[M+H]^+$	7	r^2>0.997 RSD = 0.5–9.7% LOD = 7–29 ng/L	[15]
3FQs: NOR, CIPRO, ENRO	Surface water (river water) SPE (1000 mL, pH 4)	Chromolith Performance RP-18e (100 × 4.6 mm)	0.025 M H_3PO_4 (pH 3.0 by TBA)/MeOH 960:40, isocratic	FD Exc: 278 nm Em: 450 nm	16	r^2>0.994 LOQ = 25 ng/L	[16]
9FQs: CIPRO, ENRO, FLE, FLU, LOME, MOXI, NOR, OFLO, OXO	Surface water, municipal wastewater, WWTP effluent, sewage sludge,	ID YMC-Pack Pro C18 (250 × 4.6 mm, 3 μm)	50 mM FAc/MeOH 78.5:21.5, gradient	FD Exc: 278–320 nm Em: 365–500 nm ESI-MS	40	r^2>0.9989 RSD <5% LOQFD = 11–60 ng/L LOQMS/MS = 0.3–7.0 ng/L	[17]

Table 1. Continued.

Substances determined	Matrix sample preparation	Stationary phase analytical column	Mobile phase	Detection	Analysis time (min)	Validation data	Ref.
	sediment SPE (500 mL, pH 4.2)			ESI-MS/MS $[M+H]^+$			
9FQs: CIPRO, DANO, ENO, ENRO, NOR, CINO, FLU, NAL, OXO	Surface water (lake and river water) SPE (250 mL, pH 4)	Polarity dC18 (150 × 3 mm, 3 μm)	FAc (pH 2.5)/ACN 96:4, gradient	UV 275 or 255 nm	25	r^2>0.99 LOD = 8–20 ng/L	[25]

a) CINO, cinoxacin; FLE, flexoxacin; GATI, gatifloxacin; MARBO, marbofloxacin; MOXI, moxifloxacin; PIP, pipemidic acid; PIRO, piromidic acid; SPAR, sparfloxacin; TOS, tosufloxacin.

detection for the separation of 20 FQs within 12 min [6]. The separation was performed on BEH C18 analytical column at acidic pH. Despite very fast separation of 20 analytes, the disadvantage of this method was spending a lot of time for sample percolation during SPE step. Study of Tamtam *et al.* employed UHPLC-MS/MS for the determination of 17 antibiotics from different groups within 10 min [20]. However, no internal standard has been used for the quantification which is fully recommended for the analysis of environmental samples. Another study for the determination of 21 antibiotics from seven different classes by UHPLC-MS/MS at acidic pH was developed [21]. The analysis was performed within 10 min; however, authors spent about 80 min during SPE procedure.

Recently, Waters Corporation has designed a new column manager with the possibility of connection of four independent analytical columns into UHPLC system simultaneously. These columns can be switched automatically by means of a switching valve, enabling the possibility to perform fully automated systematic method development approach in one sequence. In our study, four analytical columns of different chemistries (reverse-phase C18 column BEH C18, C18 column BEH Shield RP18 with embedded polar phase, phenyl–hexyl analytical column BEH Phenyl, and silica-based analytical column HSS T3 C18) were tested. Two organic modifiers (methanol (MeOH) and ACN) and two buffers (acidic and basic) (*e.g.* pH 3 and 9) could be tested in gradient mode of elution. Using generic gradient within 5 h, 14 different chromatograms including all variables (column, buffer and organic modifier) were generated. Prearranged setting of "systematic method development" was developed and recommended by Waters Corporation as universal technique. Of course, any step (analysis conditions, wash step) and any input (column, buffer and organic modifier) can be modified upon request of the developed method. After series of measurement, the software is able to interpret results into reports with criteria set by the user (*e.g.* injection score report, peak capacity report, total peak with resolution higher than 1.5 report), allowing the final consideration for the selection of analytical column, organic modifier and pH of analysis. For example, total peak number report shows the number of integrated peaks in each separation (Fig. 2). Such preliminary results for all variables facilitate further method development and fine tuning.

In this study, conventional approach and systematic method development for the determination of five commonly used FQs were compared. Initially, the separation of FQs was performed at acidic pH according to the previously published results [4]. On the other hand, the separation of FQs at basic pH at values higher than pK_{a2} has never been performed probably because of low pH stability of conventionally used silica-based analytical columns. Performance and sensitivity of UHPLC method using both FD and MS detection were compared. The developed UHPLC-FD method was used for the evaluation of stability of FQ solutions, whereas developed UHPLC-MS/MS method was applied for the analysis of wastewater samples.

2 Materials and methods

2.1 Reagents and materials

Reference standards of FQ antibiotics (ofloxacin (OFLO), pefloxacin (PEFLO), CIPRO, NOR, ENRO and NOR d_5 – isotopically labelled) were obtained from Sigma-Aldrich (Prague, Czech Republic). All pharmaceuticals were of analytical grade (purity ≥98%). The ammonium acetate (AmAc), ammonium formate (AmF), formic acid (FAc), the ammonia, all reagent grade, ACN, HPLC-gradient grade and MeOH, LC-MS grade, were purchased from Sigma-Aldrich. HPLC-grade water was prepared by Milli-Q reverse osmosis Millipore (Belford, MA, USA) and it meets European Pharmacopoeia requirements.

2.2 Sample collection

Briefly, 24-h composite samples of influent and effluent after chlorination were collected from WWTP in the University Hospital in Hradec Králové in September 2009. The WWTP is a mechanical–biological sewage treatment plant with complete aerobic sludge stabilization (hydraulic retention time is approximately 8 h). The maximum daily influx of the mechanical and biological part is 250 m^3/day and it depends on the activity of the individual hospital departments. The main task of this wastewater plant is to remove the dangerous property of the wastewater infectivity. Treated wastewater flows directly to the municipal WWTP for the whole city, Hradec Králové. River samples were collected from the river Elbe in Hradec Králové. The samples were stored in 2-L glass bottles in fridge at 4°C and analyzed as soon as possible.

2.3 Preparation of standards, fortified samples and blank samples

Stock solutions of FQs were prepared at a concentration of 0.1 mg/mL in MeOH and they were stored in a glass vial at 4°C in dark. Working standard solutions were prepared in a mixture of AmAc, pH 10.5, and MeOH (9:1, v/v) by appropriate dilution of stock solution.

The water samples were filtrated through 0.2-μm filters to remove solid particles. To obtain fortified samples, 20 mL of water samples were spiked with antibiotic standard mixture to obtain final low-, medium- and high-concentra-

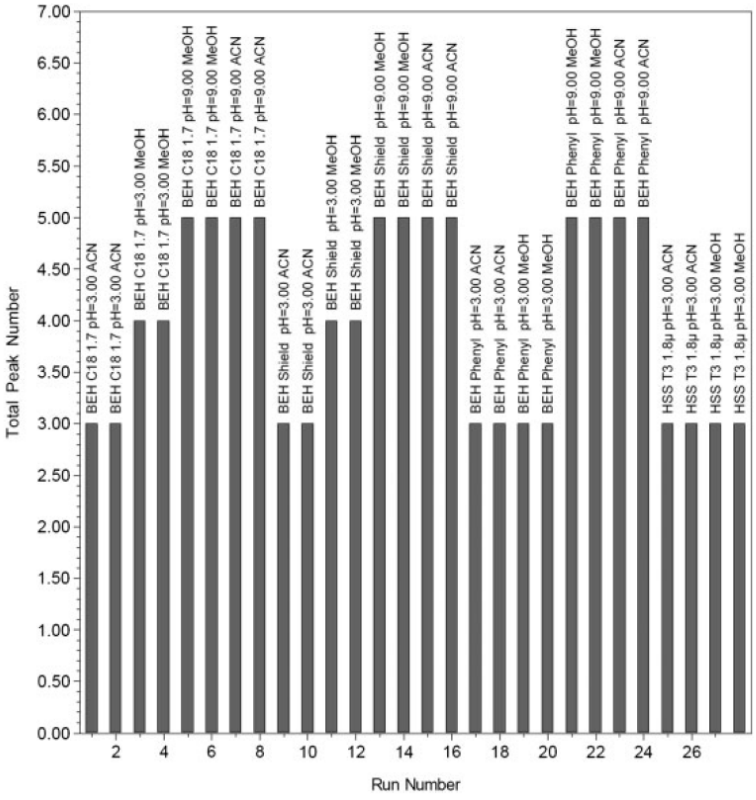

Figure 2. An example of report generated by Empower software. Total peak number report shows the number of separated peaks in each chromatogram.

tion level. Internal standard NOR-d_5 was added to each sample before sample preparation and to the mixture of standards of FQs. All samples (spiked samples and blank samples) were adjusted to pH 10.5 with ammonia and 0.02 g of EDTA was added to avoid antibiotics to form complexes with divalent ions and metals and to avoid possible sorption to, *e.g.* glass surface.

2.4 SPE

A completely new extraction procedure for the preconcentration of FQs and cleanup at basic pH 10.5 was developed. Water samples were extracted through Oasis HLB cartridges (6cc/200 mg, Waters, Prague, Czech Republic). The cartridges were activated with 3 mL of MeOH and conditioned with 3 mL of water, pH 10.5. After sample percolation, the SPE cartridges were washed with 3 mL of 10% MeOH in water, 3 mL of 2% acetic acid and 12 mL of water. After being washed, HLB cartridges were dried and subsequently analytes were eluted with 2 mL of 1% ammonia in MeOH. The eluate was evaporated to dryness under a gentle stream of nitrogen and redissolved in 0.5 mL of mixture of 10 mM AmAc, pH 10.5, and MeOH (9:1). The sample was further sonicated for 30 min and vortexed for 1 min. Finally, the sample was filtered through 0.2 μm PTFE filter to remove possible particles and injected onto UHPLC system. An achieved enrichment factor was 40.

Cleanup efficiencies were studied in order to monitor the effect of pH on retention of FQs. The effects of the type and pH of solvents used during conditioning and washing step and elution were evaluated.

2.5 LC and MS

2.5.1 LC

Two UHPLC Acquity (ACQ) chromatographic systems were used in this study (Waters). UHPLC for systematic method development and for UHPLC-FD measurements consisted of ACQ-binary solvent manager, ACQ-sample manager, ACQ-column manager, ACQ-PDA detector and ACQ-FLR detector. During the systematic method development ACQ BEH C18, ACQ BEH Shield RP 18, ACQ BEH Phenyl and ACQ HSS T3 C18 columns all in 50 × 2.1 mm, 1.7 or 1.8 μm dimensions were used. They were all obtained from Waters. The columns were maintained at 30°C at a flow rate 0.6 mL/min. The injection volume was 2 μL. The gradient elution was used for the separation. MeOH or ACN was used as the organic modifier (solvent A) and 10 mM AmF buffer (pH 3.0) or 10 mM AcAc buffer (pH 9) was used as aqueous component of mobile phase (solvent B). The gradient conditions were initiated with 5% A followed by a linear increase to 95% A in 5 min and by re-equilibration for 2 min. The design of systematic method development was as follows: First, each column was equilibrated for 2 min. Subsequently, one blank (initial mobile phase) and two samples (mixture of FQs) were injected at four different gradients using ACN/buffer, pH 3, MeOH/buffer, pH 3, MeOH/buffer, pH 9, and ACN/buffer, pH 9, always after 2 min of equilibration. After a set of experiments, each column was washed with ACN for 2 min and ACQ-CM automatically switched to the next column. This process was repeated on BEH C18, BEH Shield RP 18 and BEH Phenyl, whereas on HSS T3 C18 only two gradients using ACN/buffer, pH 3, and MeOH/buffer, pH 3, could be performed because of limited stability of silica-based column. The results showed better separation of FQs at pH 9. Thus, in second set of experiments, AmAc solution at pH 10.5 and widely used 0.1% FAc (solvent B) were tested.

The measurements of fluorescence spectra at acidic pH 3.0 and basic pH 10.5 were performed in order to check the optimal excitation and emission wavelengths. Moreover, the comparison of FD sensitivity at commonly used $\lambda_{exc} = 278$ nm and $\lambda_{em} = 450$ nm and the optimal excitation and emission wavelengths found at acidic and basic pH were accomplished.

The gradient separation at basic conditions resulting from systematic approach was further optimized. The separation of FQs using UHPLC-FD was finally enabled using ACQ BEH Phenyl (50 × 2.1 mm, 1.7 μm). The column was maintained at 35°C at a flow rate 0.45 mL/min and the injection volume was 2 μL. MeOH (solvent A) and 10 mM AmAc pH 10.5 (solvent B) were used as mobile phases. The gradient conditions were initiated with 5% A kept for 1 min followed by linear increase to 75% A in 3 min. During next 4 min, the concentration of solvent A was decreased to 45% and then followed re-equilibration to initial conditions. The fluorescence detector was operated at 278 nm excitation and 450 nm emission wavelengths based on our previously published method [4] and the sensitivity was compared with the measurement at $\lambda_{exc} = 310$ nm and $\lambda_{em} = 415$ nm, which were fluorescence maxima found at basic pH.

The comparison of sensitivity at acidic pH was performed as well. Separation at acidic pH was achieved based on our previous experience using ACQ BEH C18 (50 × 2.1 mm, 1.7 μm) at 30°C under isocratic condition at a flow rate of 0.3 mL/min. The mobile phase was a mixture of 10 mM AmF (pH 3) and MeOH (81:19, v/v). The measurements were carried out in two series because of impossibility to separate all five FQs sufficiently. In first set, OFLO, NOR and ENRO were measured. PEFLO and CIPRO were measured in second set of experiments. The detector was operated at 278 nm excitation and 450 nm emission wavelengths. For the comparison of FD sensitivity, the detection was performed at excitation 310 nm and emission wavelengths 447 nm, which were optimal values at acidic pH.

Second UHPLC system consisted of ACQ-binary solvent manager, ACQ-sample manager and ACQ-TUV detector. It was coupled to Quattro Micro triple quadrupole mass spectrometer (Micromass, Manchester, GB, UK) equipped

with a multi-mode ionization source (ESCI). Separation of FQs was achieved by an ACQ BEH Phenyl analytical column (50 × 2.1 mm, 1.7 μm). The column was maintained at 35°C at a flow rate 0.35 mL/min and the injection volume was 2 μL. MeOH (solvent A) and 0.5 mM AmAc pH 10.5 (solvent B) were used as a mobile phase. The gradient conditions were initiated with 2% A followed by linear increase to 45% in 2 min. During next 0.5 min, the concentration of solvent A was further increased up to 70% following by re-equilibration for 2 min to initial conditions.

2.5.2 MS

ESI positive mode was used for analysis. Ion source was set up was as follows: capillary voltage: 1000 V, ion source temperature: 130°C, extractor: 2.0 V and RF lens: 0.2 V. The desolvation gas was nitrogen at a flow rate of 500 L/h and the temperature 450°C. Cone voltage was set up individually for each analyte (Table 2). Nitrogen was also used as a cone gas (100 L/h) to prevent the contamination of sample cone. Quantitation of all analytes was performed using selected reaction monitoring (SRM) experiment. Two specific SRM transitions were optimized for each analyte in order to increase selectivity of the method. Argon was used as a collision gas and collision energy was optimized for each analyte individually as summarized in Table 2. The MassLynx 4.1 software was used for MS control and data gathering. QuanLynx software was used for data processing and quantification–regression analysis of calibration curves and calculation of concentrations.

2.6 System suitability test and validation

Identification of the target FQs was accomplished by a comparison of retention times (UHPLC-FD) and using the two SRM ion transitions for each compound (UHPLC-MS/MS). The first SRM transition was utilized for quantitation and the second for confirmation. Moreover, secondary ion ratio of the two SRM ion transitions was compared with those of standards for unequivocal confirmation. Difference within 20% was considered as an agreement. In order to compensate for the loss of target analytes during the extraction procedure and account for the matrix effects, an internal standard NOR-d_5 was spiked to the samples prior to extraction.

An important part of method validation is the system suitability test (SST), details of which are usually given in Pharmacopoeias [22]. The SST was performed under optimized chromatographic conditions. Resolution, asymmetry factor, repeatability of retention time and peak area were tested in UHPLC-FD method. UHPLC-MS method was checked only for the repeatability of retention times and peak area.

Calibration curves of all analytes in the concentration range of 0.01–5 μg/mL for UHPLC-FD were measured. The concentration range used for UHPLC-MS/MS method was from 0.5 to 500 ng/mL, corresponding to concentrations in water samples ranging between 0.0125 and 12.5 μg/L taking into account the 40-fold concentration factor of the SPE. Calibration curves were generated by linear regression of peak areas of the standard solutions against their respective concentrations. LODs and LOQs were established based on S/N. LOD was expressed as $S/N = 3$ and LOQ was expressed as $S/N = 10$.

Method precision and accuracy were established to evaluate the SPE procedure. For the precision, spiked wastewaters at three different concentration levels were measured in three replicates to calculate RSD, which describes the closeness of agreement between series of measurements. Accuracy was determined as a method recovery using spiked wastewaters, again at three different concentration levels in three replicates in order to establish the closeness of agreement between the true and the measured value as it corresponds to International Conference on Harmonization requirements [23]. Recoveries were calculated by comparison of the peak areas obtained from spiked samples with the peak areas from the same samples without standard solution addition (blanks), finally, with the areas obtained by direct injection of a standard solution at

Table 2. SRM transitions for all analytes and MS/MS parameters

Compound	Precursor		Fragment		Dwell time	Cone *V*	Collision *E*	SRM ratio	t_R
NOR	319.9	$[M+H]^+$	301.9	$[M+H-H_2O]^+$	0.05	30	20	3.4	1.25
			276.2	$[M+H-CO_2]^+$	0.05	30	15		
NOR-D5	324.9	$[M+H]^+$	307.0	$[M+H-H_2O]^+$	0.05	30	20	4.0	1.24
			281.1	$[M+H-CO_2]^+$	0.05	30	15		
CIPRO	331.9	$[M+H]^+$	314.0	$[M+H-H_2O]^+$	0.05	25	20	1.1	1.37
			230.5	$[M+H-H_2O-C_2H_5N-C_3H_4]^+$	0.05	30	35		
PEFLO	333.9	$[M+H]^+$	289.9	$[M+H-CO_2]^+$	0.05	30	15	1.5	1.62
			316.3	$[M+H-H_2O]^+$	0.05	30	20		
OFLO	362.0	$[M+H]^+$	261.2	$[M+H-H_2O]^+$	0.05	30	25	2.6	1.71
			318.0	$[M+H-CO_2]^+$	0.05	30	20		
ENRO	359.9	$[M+H]^+$	316.1	$[M+H-CO_2]^+$	0.05	30	20	3.5	2.00
			341.9	$[M+H-H_2O]^+$	0.05	30	20		

the concentration level expected after sample treatment. The method was considered accurate if recoveries were in the range 80–120% and precision was satisfactory if RSD was lower than 20%.

3 Results and discussion

3.1 Conventional method development

The analysis of four FQs including NOR, CIPRO, OFLO and ENRO using HPLC-FD performed on monolithic column was previously published by our group [4]. Further, PEFLO was added into analytical method as it is used for the treatment in Czech Republic as well. This became a challenge, because the separation of OFLO and PEFLO at low pH on C18 was difficult even impossible because of similar retention on analytical column and consequently the elution at same retention times. Although various gradient elutions employing different additives were used, the separation with sufficient resolution of those analytes has not been achieved. Thus the coupling of HPLC with fluorescence detector could not be used for separation of all five FQs anymore.

Consequently, further experiments were performed on UHPLC system. A new method was developed on UHPLC-MS/MS using BEH C18 analytical column. Mobile phase employed MeOH in combination with volatile additives including FAc, AmAc or AmF buffers at acidic pH, which is in accordance with the previously published results dealing with the analysis of FQs (Table 1). However, the results obtained were not repeatable. The peak shapes at low pH were also not satisfactory, probably due to low concentrations of additives. Therefore, systematic method development was employed in order to re-optimize the method properly.

3.2 Systematic method development

For the systematic method development, the prearranged settings developed and recommended by Waters Corpora-

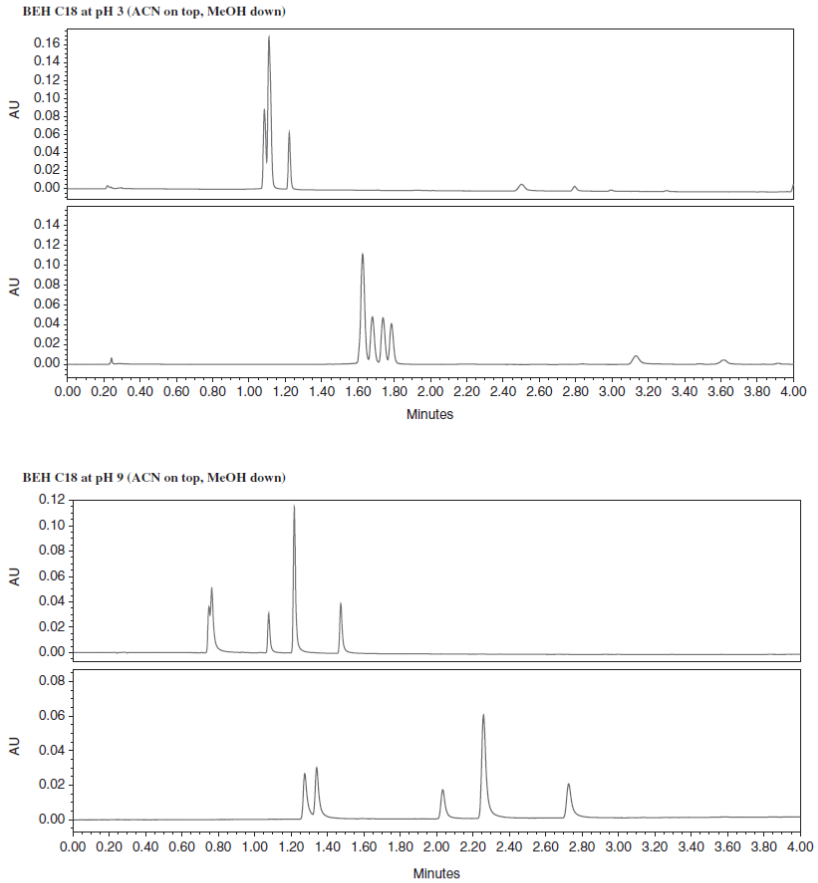

Figure 3. The influence of organic modifier and pH on separation of FQs on C18 analytical columns, conventionally used in FQs analysis.

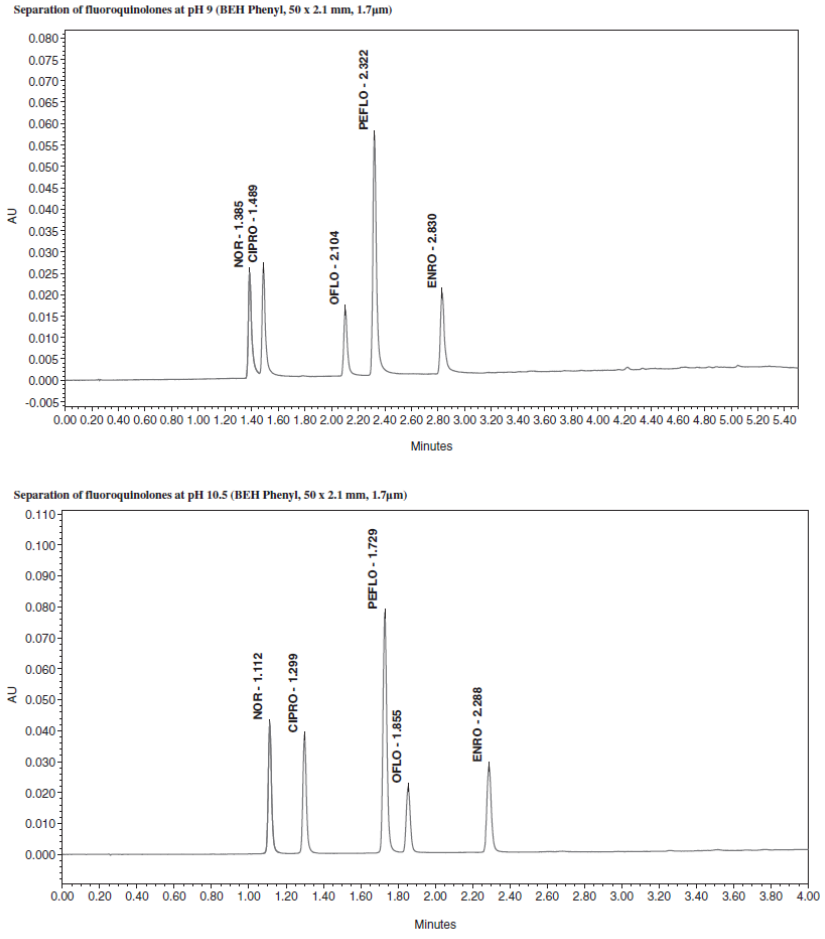

Figure 4. Chromatogram of standard solution at pH 9 (upper) and at pH 10.5 (lower) obtained on UHPLC system. The peak order switch is demonstrated in dependence on relatively small change of pH.

tion, described in Section 2.5, were used. C18 analytical column seems to be a standard in the analysis of FQs. Therefore, it was chosen for the first judgment of organic modifier and pH choice. As shown in Fig. 3, MeOH was substantially more efficient in the separation of FQs than ACN and it was used for further method development. Second, pH was found to be a crucial factor for FQs separation as they are zwitterions and their ionization is pH dependent. At pH 3, which is the pH usually used in FQs analysis [4, 15], the separation of two peaks (NOR and CIPRO) was insufficient. Contrary to all the published results of separations performed at acidic pH, much better separation was obtained at pH 9 on all tested columns BEH C18, BEH Phenyl and BEH Shield RP 18 (chromatographic data not shown, see report in Fig. 2). HSS C18 must have been excluded from pH 9 experiments as it is silica-based column and thus its pH range is limited. Moreover, the elution order of FQs at pH 9 was changed as follows: NOR, CIPRO, OFLO, PEFLO, ENRO in contrast to elution order at acidic pH which was OFLO, PEFLO, NOR, CIPRO and ENRO. The best resolution of the first two peaks – NOR and CIPRO – was observed on BEH Phenyl column. However, slight peak tailing was still observed. In order to improve peak shape and the resolution of the first two peaks, higher pH of 10.5 was tested in further experiments. Surprisingly, such relatively small change in pH (about 1.5 unit) was again crucial in FQs separation. As it was expected, the peak shape improved and there was no more tailing observed. In addition, the resolution between the peaks of NOR and CIPRO was substantially improved (Fig. 4). Moreover, pH 10.5 also caused the switching of the order of OFLO and PEFLO and final order of separated peaks was as follows: NOR, CIPRO, PEFLO, OFLO and ENRO. Subsequently, BEH C18 and BEH Shield RP18 analytical columns were no more suitable for the analysis of tested FQs as the peaks of OFLO and PEFLO were not baseline separated any more (Fig. 5). BEH Phenyl showed the best separation of critical pairs of peaks NOR/CIPRO

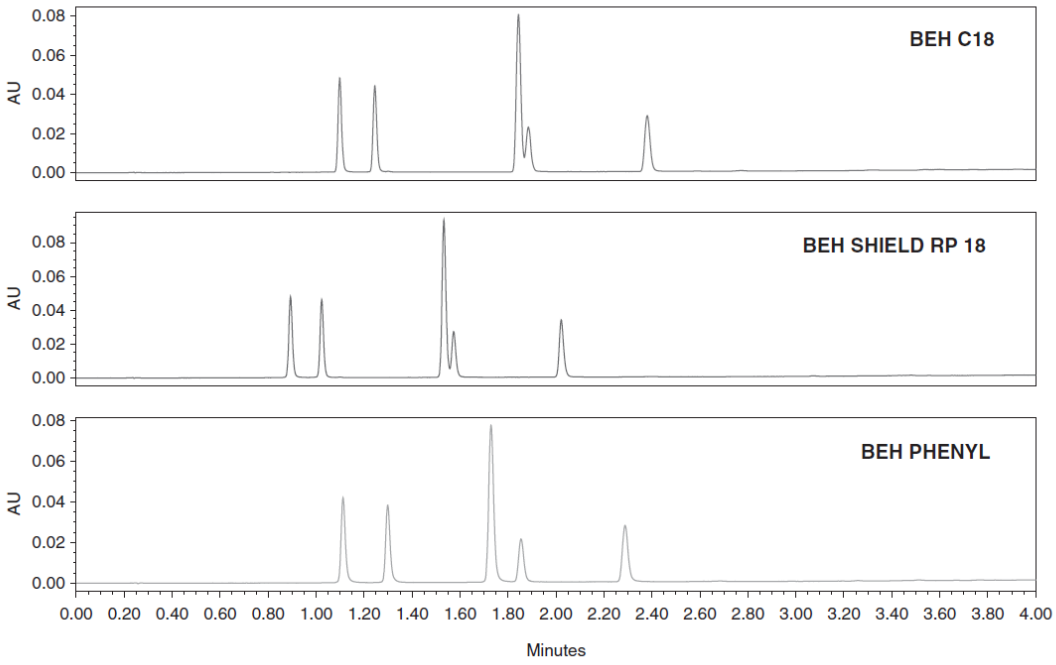

Figure 5. Chromatograms obtained during systematic method development on different stationary phases. Mobile phase composition: MeOH and AmAc pH 10.5.

and OFLO/PEFLO. Therefore, it was used in further study for the development of UHPLC-FD as well as for UHPLC-MS/MS method.

Using systematic method development revealed that FQs can be successfully separated at pH 10.5 on BEH Phenyl (50 × 2.1 mm, 1.7 μm) analytical column. Recently, there was a published study dealing with the stability of analytical columns [24]. They reported the unstability of XBridge phenyl analytical column (Waters) over pH 7. However, in this study, we have not observed any changes of peak shapes of retention times during usage of BEH phenyl analytical column at basic pH 10.5 even after 3000 injections (approx. 2000 injections at basic pH).

3.3 Optimization of UHPLC analysis with FD and MS detection

3.3.1 UHPLC-FD

The gradient profile and mobile phase flow rate used in the systematic method development were further optimized in order to keep baseline separation of all FQs and to shorten the analytical run. The separation of critical pairs NOR/CIPRO and PEFLO/OFLO in one mixture is an analytical challenge. The previously published studies using FD typically did not include combination of all these four analytes, especially PEFLO has not been included in analysis.

Most of the studies utilized low pH, as fluorescent properties of FQs are more advantageous (Table 1). In our study, commonly used excitation and emission wavelengths, 278 and 450 nm, were used for the detection at acidic pH [3, 4, 14, 15]. However, fluorescence spectra measurements were performed in order to compare optimum excitation and emission wavelengths at acidic pH 3.0 and basic pH 10.5. The difference of fluorescence spectra could be observed in dependence on pH of mobile phase. CIPRO, PEFLO and ENRO showed the same fluorescence spectra. At acidic pH, they showed the maxima of λ_{exc} = 310 nm and λ_{em} = 447 nm, whereas at basic pH the optimal λ_{exc} and λ_{em} wavelengths were 310 and 415 nm, respectively. NOR revealed similar spectra; however, small differences from CIPRO, PEFLO and ENRO could be observed. The optimal λ_{exc} and λ_{em} at acidic pH were 311 and 447 nm, whereas at basic pH these optimal λ_{exc} and λ_{em} were 308 and 415 nm, respectively. OFLO showed completely different spectra from the other FQs. At acidic, λ_{exc} = 298 nm and λ_{em} = 490 nm, whereas at basic pH the optimal λ_{exc} and λ_{em} wavelengths were 295 and 464 nm, respectively. Consequently, further assay comparing the sensitivity of FD of FQs at commonly used λ_{exc} = 278 nm and λ_{em} = 450 nm and the optimal wavelengths was carried out and the results revealed no significant difference of sensitivity at the same pH. However, 10- to 50-fold higher sensitivity of FD at acidic pH 3.0 than at basic pH 10.5 was observed (Table 3). Our results were in agreement with the results of Schneider *et al.*

Table 3. SST and validation data for UHPLC-FD method and UHPLC-MS/MS method at basic pH 10.5[a)]

	UHPLC-FD at basic pH					UHPLC-MS/MS				
SST	NOR	CIPRO	PEFLO	OFLO	ENRO	NOR	CIPRO	PEFLO	OFLO	ENRO
Resolution	–	1.71	5.52	2.86	6.61					
Asymmetry factor	1.08	1.04	1.02	0.97	1.05					
t_R	2.49	3.07	3.89	4.31	5.23	1.25	1.37	1.62	1.71	2.00
Repeatability t_R (% RSD)	0.14	0.13	0.10	0.10	0.04	0.00	0.00	0.20	0.00	0.26
Repeatability A (% RSD)	0.52	1.00	0.26	1.10	0.81	3.19	4.23	4.55	4.04	4.56
Validation										
Linearity (r^2)	0.9998	0.9995	0.9990	0.9991	0.9999	0.9990	0.9989	0.9996	0.9994	0.9989
Linearity–range (ng/mL)	150.00–5000.00	100.00–5000.00	50.00–5000.00	50.00–5000.00	100.00–5000.00	1.00–500.00	1.00–500.00	5.00–500.00	0.50–500.00	0.50–500.00
IDL (pg injected into column)						1.00	1.00	2.00	0.20	0.20
LOD (ng/mL)	50.00	25.00	25.00	10.00	25.00	0.50	0.50	1.00	0.10	0.10
LOQ (ng/mL)	150.00	100.00	50.00	50.00	100.00	1.00	1.00	5.00	0.50	0.50
Tested levels (L1, L2 and L3) (ng/mL)	250, 1000, 2500	250, 1000, 2500	250, 1000, 2500	250, 1000, 2500	250, 1000, 2500	50, 100, 250	50, 100, 250	50, 100, 250	50, 100, 250	10, 50, 100
Recovery L1 (%)	128.95	606.57	79.11	88.97	88.99	100	102	92	100	102
Recovery L2 (%)	108.65	292.00	63.56	75.21	74.66	99	100	119	101	102
Recovery L3 (%)	80.63	37.76	83.39	92.37	96.18	98	90	85	90	100
Intra-day precision L1 (%)	0.22	1.54	1.71	1.22	1.92	5.00	3.17	4.02	2.10	4.29
Intra-day precision L2 (%)	4.21	9.95	2.08	1.47	0.42	3.00	2.58	0.96	1.92	2.27
Intra-day precision L3 (%)	4.99	9.45	1.59	1.13	1.32	2.98	1.49	3.84	0.62	2.07
Inter-day precision L1 (%)						7.61	16.63	18.69	14.08	14.34
Inter-day precision L2 (%)						11.85	9.72	19.46	12.50	16.55
Inter-day precision L3 (%)						18.71	19.70	18.69	10.24	13.07

	UHPLC-FD at acidic pH				
Linearity (r^2)	0.9998	0.9996	0.9993	0.9999	0.9996
Linearity–range (ng/mL)	1.00–500.00	2.50–500.00	1.00–500.00	2.50–1000.00	2.50–500.00
LOD (ng/mL)	0.50	1.00	0.50	1.00	1.00
LOQ (ng/mL)	2.50	2.50	1.00	2.50	2.50
Increase of sensitivity	75 ×	300 ×	300 ×	60 ×	150 ×

a) Second part of the table shows the sensitivity of FD detection at acidic pH 3.0.

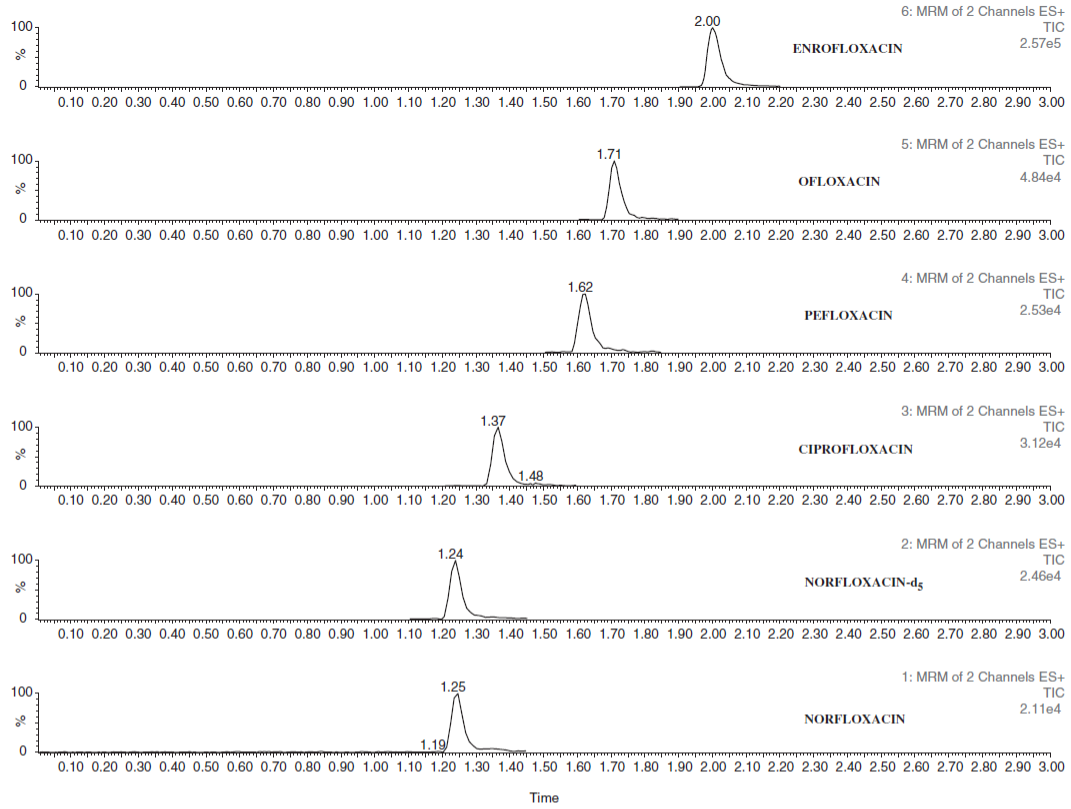

Figure 6. Chromatogram of standard mixture of FQs.

[19] who pointed out lower sensitivity of FD detection at high pH.

This newly developed UHPLC-FD method was applied only for the evaluation of stability of FQ in standard stock solution at basic pH, as FQs have never been analyzed at such conditions. The FQs were stable for 2 weeks (peak area did not decrease under 95% of initial peak area for all five FQs tested) and after this time interference near to retention time of CIPRO occurred avoiding the accurate quantitation.

3.3.2 UHPLC-MS/MS

UHPLC-MS/MS was employed for more specific determination of FQs in wastewater samples. For all FQs, protonated molecule $[M+H]^+$ gave the highest intensity signal in electrospray positive mass spectra, and therefore it was chosen as precursor ion (Table 2). The gradient used for the separation of FQs was slightly different from the generic one used during the systematic method development. The flow rate of mobile phase was decreased to 0.35 mL/min as high flow rate is not suitable for connection with ESI and the gradient elution was adjusted in order to reduce analysis time. Moreover, the optimization of mobile phase additive was performed. Several lower concentrations of AmAc pH 10.5 were tested (0.5, 1 and 2 mM) as the concentration 10 mM AmAc employed for UHPLC-FD could not be used in UHPLC-MS since signal suppression occurred. Finally, concentration 0.5 mM AmAc was used for further experiments.

Subsequently, all the parameters of mass spectrometer were tuned in order to obtain appropriate sensitivity of precursor ion for all analytes (Section 2.5). Cone voltage was set up individually for each analyte (Table 2). Finally, at optimized conditions product ions for SRM transitions were chosen according to the fragmentation pathways in Product ion scan mode. Collision energy was optimized for each analyte and for each of its two transitions individually in order to get high sensitivity (Table 2). Deuterium-labeled NOR-d_5 was used as internal standard for quantitation. At these conditions, five FQs were eluted within 2.5 min (Fig. 6).

3.4 SPE and matrix effects

As the chromatographic analysis has been performed at basic pH 10.5 in this study, a new extraction procedure had to be

developed in order to be well compatible with this pH and to achieve better compatibility of method. This new extraction procedure is contrary to all published results, where SPE was usually performed at acidic pH 3 or 4 (Table 1). Oasis HLB cartridges were chosen for the SPE because of their properties and possibility of usage at high pH.

The matrix interference is a very important problem in the environmental area. Therefore, different solvents for SPE washing step were tested in order to obtain clean extract and minimal matrix effects. First, the concentration of MeOH in milli-Q water was optimized (5, 10, 15, 20 and 30% MeOH in milli-Q water). The best recoveries were achieved with the 10% MeOH concentration. Ultra-pure water was used as a second washing solvent. However, it did not prevent the occurrence of interferences. Therefore, several acidic solvents have been subsequently tested (0.5, 1 and 2% acetic acid). The best recoveries were obtained after the percolation of 3 mL of 2% acetic acid followed by washing the cartridge with 12 mL of ultra-pure water because lower amount led to decrease of recoveries of FQs. Thus final washing step was percolation of 3 mL of 10% MeOH, 3 mL of 2% acetic acid and 12 mL of ultra-pure water.

MeOH and ACN were tested as elution solvents and MeOH showed better elution efficiency than ACN. Finally, only 2 mL of elution solvent has been found to be satisfactory. After elution, samples were evaporated to dryness and dissolved in a mixture of 10 mM AmAc pH 10.5 and MeOH (9:1). The dissolution time in order to dissolve dry extract sufficiently was another crucial issue. The best results were obtained after 30 min in ultrasonic bath followed by use of vortex for 1 min.

3.5 SST and method validation

The SST was performed by ten subsequent injections of standard mixture of FQs at a concentration of 100 ng/mL for UHPLC-MS and 1 μg/mL for UHPLC-FD. The repeatability of the injection of standard solution was established (retention times and peak areas were checked, the repeatability was expressed as RSD in %). SST results are summarized in Table 3. For both UHPLC methods, the repeatability for retention times was within 0.26% RSD. Repeatability for peak area was within 5% for UHPLC-MS and within 1.1% for UHPLC-FD method, which is fully acceptable. For UHPLC-FD further parameters including resolution and asymmetry factor were evaluated (Table 3). They were both within the limits of acceptance given by Pharmacopoeia [22].

3.5.1 Linearity–calibration range

Calibration curves of all analytes were measured in the concentration range of 0.5–500 ng/mL for UHPLC-MS to define method sensitivity. Sensitivity of UHPLC-FD was compared at acidic and basic pH and the linearity was measured in the concentration range of 0.001–0.5 and 0.05–5 μg/mL for acidic and basic pH, respectively. Correlation coefficients for all FQs were higher than 0.9990 for both methods, indicating good linearity in the tested concentration ranges (Table 3).

3.5.2 Accuracy and precision

Accuracy and precision were established by spiking wastewater samples at three concentration levels (Table 3) using SPE step described in Section 2.4. Method precision was determined as intra-day and inter-day variability of three determinations at three different levels expressed as % RSD. For UHPLC-MS intra-day precision was generally within 5% RSD, whereas inter-day precision within 20% RSD, which is fully acceptable taking into account the complexity of environmental samples. Method accuracy was determined as percentage of recovery using wastewater samples spiked with standard solutions treated by SPE at three concentration levels – results are summarized in Table 3. Recoveries related to NOR-d_5 for UHPLC-MS typically ranged from 84 to 103%.

Concerning UHPLC-FD, spiked river water treated by SPE was first evaluated. Method accuracy was expressed as percentage of recovery. The results were between 95 and 101% (data not shown). Fortification of wastewater samples with standard solutions at three concentration levels treated by SPE showed the unsuitability of UHPLC-FD for the analysis of wastewaters at developed conditions. First, the interferences with peak of CIPRO were observed as summarized in Table 3. The values of recovery were 606, 292 and 37% at three fortification levels, respectively. Only intra-day precision was established as the results of accuracy were not satisfactory. Intra-day precision was within 10% RSD. This is in agreement with study of Lee *et al.* [14] who pointed out the unsuitability of fluorescence detector for the analysis of wastewaters. Thus wastewater samples were analyzed only by UHPLC-MS/MS.

In order to verify the absence of potential interferences around the retention time of FQs and to assess the specificity of the method, the determination of matrix effects was performed. Wastewater sample treated by SPE was injected into the UHPLC system and standard solution was injected using direct infusion. At the retention times of FQs, neither negative nor positive peaks signifying the matrix effects were observed.

LODs and LOQs were calculated based on S/N. They were established first using standard solutions in mobile phase by the injection of the smallest amounts which provide $S/N = 3$. The results for UHPLC-FD and UHPLC-MS/MS methods excluding concentration factor are summarized in Table 3. As UHPLC-FD was not found convenient for the evaluation of wastewater samples at basic pH, only UHPLC-MS/MS was further used for their determination. UHPLC-MS/MS method had appropriate sensitivity to perform the determination of FQs in wastewater samples reaching LOQ

12.5–125 ng/L with a sample volume of only 20 mL. Such low volume needed for sample preparation is not typical, as previously published studies used volumes ranging typically from 250 to 1000 mL (Table 1). Large volumes of samples could be inconvenient for routine sample preparation, because the filtration and percolation of such high volume on SPE cartridges is very time consuming as the reported percolation flow rates are usually of about 3–5 mL/min. In study of Li *et al.* they developed very sensitive UHPLC-MS/MS method; however, they spent more than 80 min for percolation of 250 mL of sample during SPE procedure [21]. Another previously published UHPLC-MS/MS method [6], which developed ultra-fast separation, however, spent also up to 80 min on SPE using sample volumes of 200, 400 or 800 mL of environmental waters achieving the same detection limits as our method which used only 20 mL of wastewater samples. Using high volume of sample can lead to sensitive method if the concentration factor is high; however, spending lot of time on SPE is not suitable. The LODs of antibiotics in study of Tamtam *et al.* [20] are comparable with ours even though they used higher volume of sample (100 mL) and spent more than 30 min during the SPE procedure. For the purpose of analysis of wastewaters, such preconcentration using 20 mL of sample was sufficient, achieving similar LODs as other methods using high volumes of water samples. Moreover, comparing the instrumental LODs expressed as amount of picogram injected into analytical column, this newly developed UHPLC-MS/MS method at basic pH is more sensitive than the others that have already been published [20, 21].

The comparison of sensitivity of MS and FD detection was done. Developed UHPLC-MS/MS method was about two orders of magnitude more sensitive than UHPLC-FD at basic pH. The sensitivity of UHPLC-MS/MS was comparable to UHPLC-FD at acidic pH which is in agreement with the study of Lee *et al.* [14].

3.6 Applicability of the method to real samples

The river water samples and samples from WWTP were analyzed by UHPLC-MS/MS with SPE at pH 10.5. No antibiotics were detected in river water samples. A total of 18 samples of influent and effluent from WWTP were analyzed and NOR, CIPRO and OFLO were detected in influent sample in average concentrations of 38.11, 2468.70 and 20.27 ng/L, respectively, and only NOR and CIPRO in effluent sample in concentration levels of 25.69 and 38.06 ng/L, respectively. The CIPRO concentration detected in influent was very high compared with other studies where usually OFLO was the dominant antibiotic [6, 14].

Comparing the concentrations in influent with those in effluent, we evaluated the efficiency of treatment process. OFLO was detected only in influent sample and thus efficiency of treatment process could not be evaluated. However, from the low concentration of 20.27 ng/L detected in influent and no detection in effluent sample, its elimination can be concluded during treatment process. The elimination ratios for NOR and CIPRO were 32 and 98%, respectively. These results are consistent with those reported earlier [14, 21].

4 Concluding remarks

A new fast UHPLC method for the determination of five FQ antibiotics was developed. Conventional and systematic method development approaches were compared. During the systematic approach, a possibility of automatic switching among four independent analytical columns of different chemistries (BEH C18, BEH Shield RP18, BEH Phenyl and HSS T3 C18) has been used. ACN and MeOH as well as buffers of acidic and basic pH were tested in gradient mode of elution. Surprisingly and contrary to all the previously published results, the best separation of FQs was obtained on BEH Phenyl analytical column at basic pH (10.5), showing that pH of mobile phase is crucial for further analysis of FQs.

MS and FD at basic pH were compared from the point of view of sensitivity and applicability to the analysis of real wastewater samples. MS showed two orders of magnitude higher sensitivity and convenience for the analysis of wastewaters at basic pH than FD which showed some interferences avoiding accurate quantification of analytes. A new SPE procedure was successfully developed at basic pH using volume of only 20 mL of wastewater sample. The method was validated in terms of linearity, precision and recovery using spiked wastewater samples. Calculated recoveries ranged from 84 to 103% in wastewaters, which is fully acceptable considering the complexity of matrix. The LOQs were found to be low enough to determine FQ antibiotics in hospital wastewaters (LOQ = 12.5–125 ng/L). Therefore, the method could be applied to analyze samples from WWTP.

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The authors have declared no conflict of interest.

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5.8. Supplement IV

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Tetracycline antibiotics in hospital and municipal wastewaters: a pilot study in Portugal.

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PAPER IN FOREFRONT

Tetracycline antibiotics in hospital and municipal wastewaters: a pilot study in Portugal

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Abstract This study investigated the occurrence of tetracyclines (TCs), namely minocycline (MIN), TC, and its epimer epitetracycline (ETC), and doxycycline (DC), in four hospital wastewater effluents and its fate in municipal wastewater treatment plants (WWTPs), in Coimbra, Portugal. Analytical determination was carried out by solid-phase extraction followed by liquid chromatography with fluorescence detection. A gradient system with a mobile phase containing oxalic acid 0.02 M and acetonitrile was used. After postcolumn derivatization with magnesium reagent, TCs were detected at λ_{exc} 386 nm and λ_{em} 500 nm. The proposed method allowed good sensitivity, accuracy, and precision. LOQs were 0.5 μg l^{-1} for ETC and TC and 15 and 5 μg l^{-1} for MIN and DC, respectively. The recovery values ranged between 66.4% and 117.1%, and intraday and interday repeatability was lower than 6.8%. The method was successfully used to determine the presence of the above-mentioned TCs in 24 wastewater composite samples obtained from hospital effluents and from influent and effluent of the WWTP located in Coimbra, Portugal. MIN and TC were found in 41.7% of the samples; ETC and DC were found in 25% and 8.3% of the samples, respectively. The levels found ranged from 6 to 531.7 μg l^{-1} in hospital effluents, while its concentrations in WWTP ranged from 95.8 to 915.3 μg l^{-1}. A seasonal influence in the concentrations found has also been observed, the levels found in samples collected during spring being higher than those observed in samples collected during autumn; however, these are only preliminary results. The WWTP removal rate ranged between 89.5% and 100%.

Keywords Tetracyclines · SPE · LC–FD · Postcolumn derivatization · Environmental wastewaters

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Introduction

Pharmaceuticals are of scientific and public concern as they are recognized as a new class of environmental pollutants [1]. In Europe, monitoring of human pharmaceutical products in river watersheds is required within the framework of the European Union Water Framework Directive (EU WFD) that aims to protect and improve the quality of all water resources throughout Europe [2].

Antibiotics are among the emerging contaminants in water because of the concern of their potential adverse effects on human health, mainly due to the increased bacteria resistance on the ecosystem and on humans [3].

Generally, tetracycline (TC) antibiotics are prescribed in human medicine between 300 and 1,000 mg day^{-1} [4]. After TC administration, a significant amount is excreted as active metabolites and discharged into municipal wastewater systems [5]. As these compounds are still active, the ultimate fate of these antibiotics is an important environmental issue.

Moreover, it is expected that hospital effluent will contribute to some extent to the pharmaceutical load in the influent of the wastewater treatment plants (WWTPs), but the question is how significant this contribution is [6]. Antibiotic concentrations calculated and measured in hospital effluents are of the same order of magnitude as minimum inhibitory concentrations for susceptible pathogenic bacteria [7].

Depending on treatment of the sewage water, different concentrations of the active compounds are found after treatment and are transported into aquatic systems [8–11]. According to the EU WFD, there is a need for additional data on the occurrence of antibiotics in streams receiving large amounts of water from local WWTPs, such as coastal rivers. Coimbra city, in Portugal, with a high number of hospitals and high rate of population, might contribute with considerable concentrations of antibiotics, namely TCs, into such streams.

Contamination profiles of hospital effluents and influents/effluents of WWTPs to water courses are essential given the limited information available and concerns raised by official organizations.

In previous studies, ciprofloxacin and enrofloxacin were detected in Mondego river, in Coimbra, Portugal, with levels ranging from 67 to 119.2 ng l^{-1} [12]. Moreover, high levels of fluoroquinolone antibiotics (up to 11,000 ng l^{-1} of ciprofloxacin) were found in Coimbra hospital effluents and in influents/effluents of local WWTPs, showing seasonal level dependence and incomplete removal by means of waste treatment [13].

The analytical methodology usually applied to TC analysis in surface water samples is based on multiresidue determination, together with other antibiotics or even other pharmaceuticals [5]. Liquid chromatography (LC) has become the technique of choice for multiclass analysis, especially when coupled to mass spectrometry (LC–MS) and tandem MS (LC–MS/MS). However, due to the complexity of the matrix, in most cases, an extraction step for sample cleanup and preconcentration is required before analysis in order to achieve the required sensitivity [14]. Capillary electrophoresis has also been reported for the determination of antibiotics in environmental samples [15].

To the best of our knowledge, there are no published data on the environmental occurrence and behavior of TC antibiotics in Portugal. Hence, the aim of this research was to determine the occurrence and fate of TCs in Portuguese WWTPs for a better understanding of the potential environmental implications.

The first main goal of this work concerned the optimization and validation of an analytical methodology for a specific and sensitive determination of TC antibiotics minocycline (MIN), TC, and its epimer epitetracycline (ETC), and doxycycline (DC) in hospital and municipal wastewaters.

The methodology used here was based on solid-phase extraction (SPE) through Oasis HLB cartridges and sensitive liquid chromatography with fluorimetric detection (LC–FD) analysis. SPE coupled with LC–FD allowed selectivity and sensitivity for monitoring TCs in the aquatic environment, representing a simple, robust, and more economic alternative than MS-based methods.

In addition, our work focused on the determination of these antibiotics in four hospital wastewaters effluents, and in influent and effluent WWTPs, in Coimbra, Portugal, in order to follow the main contamination routes, evaluate seasonal influence, and WWTP removal efficiency. Some preliminary results from field measurements are reported.

Experimental

Instrumentation

The LC system consisted of Gilson pump Models 321 and 305 (Gilson, Medical Electronics, Villiers-le-Bel, France) and an injector Model 7725 (Rheodyne, Cotati, CA) with a 100-μl loop. The fluorescence detector, an LC 305 Model instrument (LabAlliance, LabAlliance, State College, PA, USA) was used. The results were recorded on a Unipoint Gilson data system (Gilson, Medical Electronics, Villiers-le-Bel, France). The LC column used was a Hichrom Lichrosorb RP8-10 (250×4.0 mm; Hichrom Limited, Reading, UK). The mixing T-piece for derivatization reaction was directly coupled through a 30 cm (0.25 mm i.d.) tube to the detector. The reaction temperature was about 22 °C.

Reagents and preparation of standard solutions

All the solvents were of LC grade and were purchased from Carlo Erba (Italy). Oxalic acid, magnesium acetate, boric acid, potassium hydroxide, sodium hydroxide, and Na_2EDTA were of analytical reagent grade and were obtained from Merck (Germany). MIN, TC, and DC were purchased from Sigma Chemical (Spain). ETC was purchased from European Pharmacopeia (Strasbourg, France).

Double-distilled water was produced by distillation and passage through Milli-Q system (Millipore, Ireland).

SPE cartridges, Oasis HLB 6 cm^3/200 mg, were purchased from Waters Corporation (USA).

Stock standard solutions of MIN, TC, ETC, and DC, prepared individually dissolving 100 mg of each standard in methanol in a 100-ml volumetric flask, were stored at 4 °C in brown glass vials for a maximum period of 1 month. The working solutions were a mixture of the four compounds prepared by a serial dilution of the stocks and were stored in amber glass vials at 4 °C. These solutions were prepared immediately before use.

The postcolumn reagent was prepared daily, as described by Pena et al. [16]. It is important to follow the addition order of the reagents, since precipitations may occur.

The mobile phase and the postcolumn reagent were filtered through a 0.45-μm filter, under vacuum, and degassed by ultrasonication.

Chromatographic conditions and detection settings

The analytical separation was performed on a reverse-phase C_8 column using gradient elution with oxalic acid solution 0.02 M as mobile phase A and acetonitrile as mobile phase B. Mobile phase B was linearly increased from 10% to 40% B in 7 min. Then, it was linearly increased until 50% in 0.5 min and held constantly until 12.9 min. Afterwards, the initial conditions were maintained for 10 min. Flow rate was maintained at 1.0 ml min^{-1}.

To produce a highly fluorescent complex, a postcolumn reaction with magnesium acetate in pH 9.0 boric acid buffer, optimized by Pena et al. [16], was performed. This reagent was delivered at a flow rate of 0.5 ml min^{-1} using a T-piece placed between the outlet of the LC column and the detector. The fluorimetric detection was carried out at an excitation wavelength of 386 nm and an emission wavelength of 500 nm, with a spectral bandwidth of 5 nm for both excitation and emission.

Sample collection and preparation

A total of 24-h composite wastewater samples were collected from the four hospitals located in Coimbra, Portugal, and from the influent and effluent of the local WWTP, during the spring and autumn of 2007.

During transportation, wastewater samples were kept in amber glass bottles, in a cooler with ice. After delivery to the laboratory, samples were filtered with 0.45-μm glass fiber filter to remove the suspended matter and stored in the dark at 4°C until extraction, which occurred within 2 days.

Sample extraction and cleanup

The samples' pH was adjusted to 3.4 with formic acid, and Na_2EDTA was added until a final concentration of 0.02 M. Then, samples were percolated through Oasis HLB 200 mg SPE cartridges, previously treated with 5 ml of methanol, 5 ml of deionized water, and 5 ml of formic acid buffer at pH3.4. Afterwards, 250 ml of sample was passed through the cartridge, and 10 ml of a water/methanol (95:5) mixture was used for the washing step. TCs were eluted with a mixture of methanol with 1% of trifluoroacetic acid.

Before injection into the chromatographic system, the methanolic eluate was concentrated to dryness under a gentle stream of nitrogen, redissolved in 250 μl of mobile phase, and filtered through a 0.45-μm membrane filter.

Results and discussion

Optimization of HPLC conditions

Since TCs have several p*K*a values, we must consider the control of mobile-phase pH in the development of the analytical method [17]. In an acidic medium (pH 1–2.5), tetracycline molecule is totally protonated and exists in its cationic form [18]. For successful ion-pair chromatography of TCs, a strongly acidic eluent is required to ensure that the cationic forms are the predominant species, which could be paired with the oxalate ion [17]. Moreover, oxalic acid concentration has an important influence on peak symmetry.

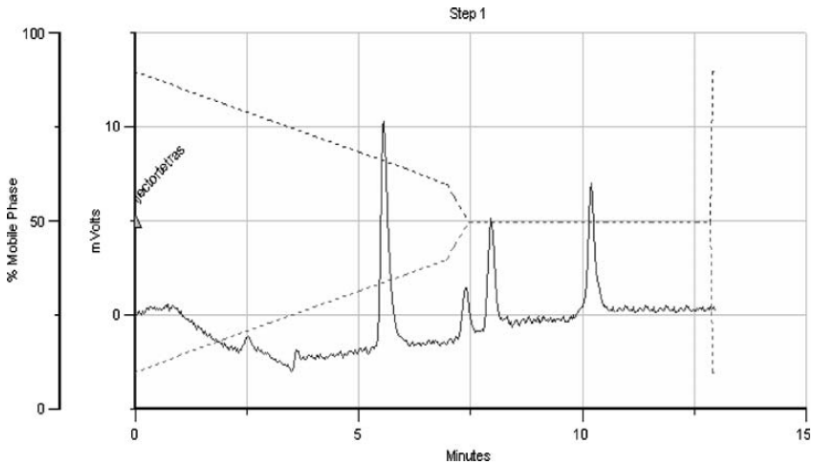

Fig. 1 LC–FD chromatogram of the standard solution of MC, ETC, TC, and DC

Table 1 Accuracy and precision validation results

Antibiotic	Levels of fortification ($\mu g l^{-1}$)	Recovery (%)	Precision intraday (%RSD)[a]	Precision interday (%RSD)[b]
Minocycline	50	86.0	3.9	5.1
	150	88.7	2.5	3.2
Epitetracycline	5.0	66.4	4.1	3.0
	25.0	72.3	3.7	2.7
Tetracycline	5.0	85.0	4.8	6.8
	25.0	91.0	3.5	3.2
Doxycycline	10.0	117.1	3.1	4.2
	50.0	89.8	5.6	4.0

[a] $n=3$
[b] $n=9$

A strong decrease in tailing factors was observed when the concentration was increased up to 10 mM [17, 19].

The methodology reported in this study uses a C_8 Nucleosil column at room temperature with acetonitrile–oxalate buffer 10 mM (20:80; pH2.0) as a mobile phase. The gradient analysis under the described conditions allows the separation of the MIN, ETC, TC, and DC with good resolution, at a flow rate of 1.0 ml min^{-1} (Fig. 1).

The presented method uses the fluorescence produced when TCs react with magnesium acetate in boric acid buffer (pH9.0), resulting in a highly fluorescent complex [16]. As mentioned above, to resolve TCs by reversed-phase LC, an acidic mobile phase must be used, but this results in quenching of the fluorescence. Therefore, post-column reagent at pH9.0 adjusts the pH for optimum sensitivity.

Postcolumn derivatization does not directly affect the chromatographic properties of TCs. However, the chemical reaction must be rapid on the chromatographic timescale to preserve the chromatographic behavior. Postcolumn derivatization also has the advantages that a separate sample treatment step is not required and that analytes are better separated from interferences prior to derivatization.

It must be emphasized that the analysis is performed at pH2.0, at which silica-based materials lack stability, and it is essential to flush the column with a neutral solvent (e.g., water–acetonitrile, 20:80) for 1 h at the end of each working day. This practice contributed positively for the column lifetime. The use of a guard column also provides some protection against decomposition of the column.

Optimization of extraction and cleanup

TCs form chelate complexes with ions at β-diketones (C_{10}-C_{12}) and carboxamide (C_2) and bind with silanol groups on C_{18} SPE sorbents which are not completely blocked [17].

Their propensity to complex with inorganic ions is, fortunately, overcome by adding Na_2EDTA, an excellent metal chelator sufficiently soluble in water, to improve recovery.

In order to validate a sensitive and accurate method, optimization of extraction and cleanup procedure was performed, namely solvents used, and their pH, in the extraction and washing steps, eluent solvents, and the volumes for eluting TCs from the cartridges.

TCs were extracted with EDTA at pH3.4 and cleaned up through Oasis cartridges. Oasis cartridges contain a macroporous polymer (poly(divinylbenzene-co-*N*-vinylpyrrolidone)) that exhibits both hydrophilic and lipophilic characteristics. The hydrophilic *N*-vinylpyrrolidone increases the water wettability of the polymer, preventing the wettability problems found with the C_{18} packings, and the lipophilic divinylbenzene provides the reversed-phase retention necessary to retain analytes [20]; moreover, the number of steps of the cleanup procedure are reduced. It contains no silanol group that simplifies the retention mechanism between sorbents and the analytes.

In this study, two amounts of this sorbent, 60 and 200 mg, with the same particle size, were evaluated. The better results, namely more clean extracts, were obtained when using 200 mg of sorbent.

Table 2 Concentrations of antibiotic residues detected in the samples analyzed, collected during autumn season

Sample Antibiotic	Hospital 1 ($\mu g l^{-1}$)	Hospital 2 ($\mu g l^{-1}$)	Hospital 3 ($\mu g l^{-1}$)	Hospital 4 ($\mu g l^{-1}$)	WWTP influent ($\mu g l^{-1}$)	WWTP effluent ($\mu g l^{-1}$)
Minocycline	nd	nd	nd	nd	350	nd
Epitetracycline	nd	18.9	nd	nd	nd	nd
Tetracycline	42.2	54.7	nd	23.2	nd	nd
Doxycycline	8.1	nd	nd	nd	nd	nd

nd not detected

Table 3 Concentrations of antibiotic residues detected in the samples analyzed, collected during spring season

Sample Antibiotic	Hospital 1 ($\mu g l^{-1}$)	Hospital 2 ($\mu g l^{-1}$)	Hospital 3	Hospital 4 ($\mu g l^{-1}$)	WWTP influent ($\mu g l^{-1}$)	WWTP effluent ($\mu g l^{-1}$)
Minocycline	317.79	531.7	nd	nd	915.3	95.8
Epitetracycline	17.5	nd	nd	6	nd	nd
Tetracycline	158	nd	nd	29.2	nd	nd
Doxycycline	nd	nd	nd	nd	nd	nd

nd not detected

Only 1 ml of 1% trifluoroacetic acid (TFA) in methanol is necessary to elute TCs from the polymeric cartridge. We have proceeded to a second elution with another 1 ml of 1% TFA in methanol, and we have not detected any peaks in the chromatogram.

Since the addition of oxalic acid to the eluent solvent was not necessary, the concentration of the eluate at low volumes was also possible.

Analytical performance

With this method, it was possible to achieve good separation and resolution for the studied TCs. On the basis of six parallel determinations, mean retention times and standard deviation (SD) for MIN, ETC, TC, and DC were 5.89, 7.63, 8.25, and 10.51 min and 0.089, 0.053, 0.08, and 0.027, respectively.

TC quantification was made with external standard method, and calibration curves were prepared using linear regression analysis and over the established range gave good fits. The mean regression coefficients (r^2) were 0.999 for MIN, 0.996 for ETC, 0.997 for TC, and 0.991 for DC.

The limit of quantification, calculated using lowering spiking assays, the LOQ being the smallest amount of the compound that can be quantified with precision (relative standard deviation (RSD) of 10%), of the proposed method was 15 $\mu g\ l^{-1}$ for MIN, 0.5 $\mu g\ l^{-1}$ for ETC and TC, and 5 $\mu g\ l^{-1}$ for DC.

The accuracy was determined by calculating the mean recovery values used for each fortification level of the wastewater samples. The accuracy was evaluated at two fortification levels for each compound. Recovery values, for all fortification levels, ranged between 66.4% and 117.1%. The precision was calculated by intraday repeatability and interday repeatability. Intraday precision (n=9) and interday precision (three nonconsecutive days) presented RSDs lower than 6.8%, demonstrating good precision. Table 1 summarizes the results obtained for the accuracy and the precision for the two fortification levels, for each antibiotic.

Application of the proposed method

Twenty-four wastewater samples, collected during spring and autumn from four hospital effluents and from influent and effluent water from a municipal WWTP, were analyzed. Tables 2 and 3 summarize the TC concentrations measured in autumn and spring, respectively. Figures 2 and 3 represent chromatograms of influent and effluent WWTP samples, respectively.

Concerning the frequency of detection, MIN and TC were found in 41.7% of the samples; 25% and 8.3% were found to be contaminated with ETC and DC, respectively.

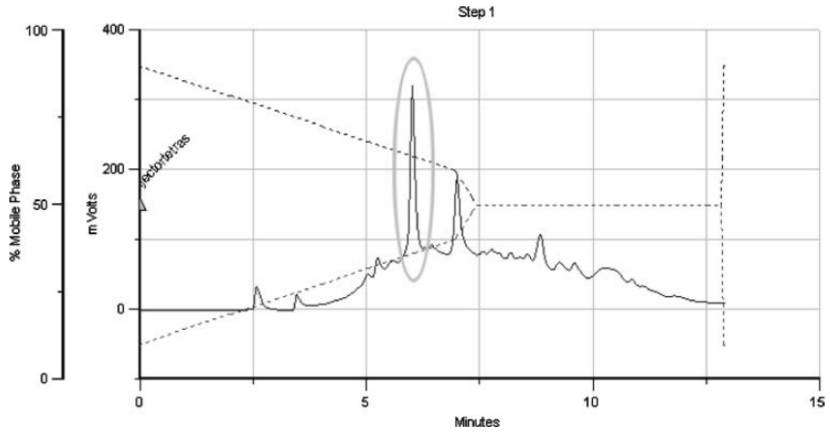

Fig. 2 LC–FD chromatogram of a WWTP influent sample

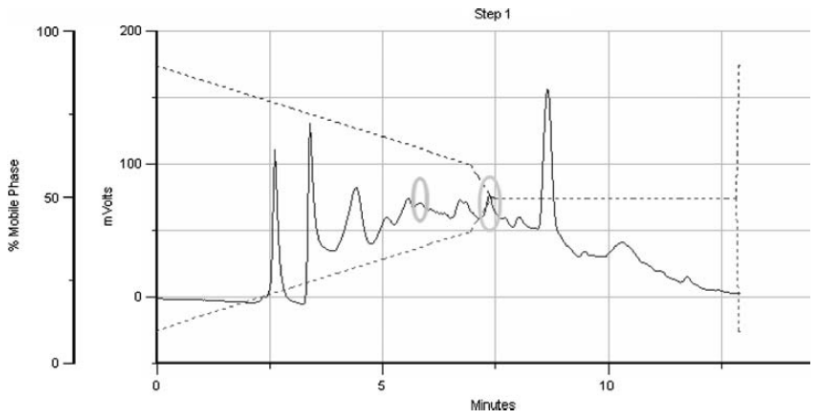

Fig. 3 LC–FD chromatogram of a WWTP effluent sample

It is expected that hospital effluents contribute to the antibiotic load in the influent entering WWTPs. To the best of our knowledge, until now, TC presence was never evaluated in hospital effluents. In the current study, it is clear that, for MIN, hospital effluent is an important point source; for the other compounds, the input from hospital is much less or negligible.

With respect to the 12 samples collected during the autumn season, differences were observed in wastewaters from the different hospitals. MIN was not present in any hospital effluent, TC was detected in three hospital effluents and one hospital effluent contained DC. The concentrations ranged from 23.2 to 54.7 μg l^{-1} for TC and 8.1 μg l^{-1} for DC. In one hospital, only TC and its epimer were detected, at a concentration of 18.9 μg l^{-1}, indicating TC degradation. In municipal wastewaters, only MIN was detected in high concentrations, 350 μg l^{-1}, and the wastewater treatment resulted in a reduction of 100%, showing the effectiveness of the treatment in the WWTP.

Regarding the 12 wastewater samples collected during the spring season, in two hospital effluents, residues of MIN were present in the highest concentrations, ranging from 317.8 to 531.7 μg l^{-1}. The second-most detected antibiotic was TC, in concentrations between 29.2 and 158 μg l^{-1}, and its epimer, ETC, was also present in a range between 6.0 and 17.5 μg l^{-1}, indicating TC isomerization due to epimer formation. DC was not detected in any sample analyzed. Once more, differences between hospital effluents were observed. One did not present any of the TCs studied, hospital 1 effluent presented MIN, ETC, and

Table 4 Overview of TCs in environmental waters in different countries

Country	Matrix	Antibiotic	Levels (μgl^{-1})	Reference
Sweden	WWTPs wastewater	Doxycycline	0.064–2.480	[26]
Luxembourg	WWTPs wastewater	TC OTC	1.0–85.0 1.0–7.0	[27]
	Rivers	TC	1.0–8.0	
		OTC	1.0–7.0	
Canada	WWTPs wastewater	TC DC	0.151–0.977 0.038–0.046	[28]
USA				
	Surface water	CTC TC	0.42–0.69 0.11	[30]
		OTC	0.34	
	WWTPs wastewater	TCs	0.05–1.14	[31]
	WWTPs wastewater	TC	0.62	[32]
(Western NY)	WWTPs wastewater	TC	0.14–0.56	[29]
(Wisconsin)	WWTPs wastewater	TC	0.05–1.2	[10]
(Colorado)	WWTPs wastewater			[33]
	Influent Effluent	TC, CTC, DC, DMC CTC	0.05–0.27 0.06	
		DC	0.07	

CTC chlorotetracycline, *DMC* demeclocycline

TC; hospital 2 effluent only contained MIN and in hospital 4 effluent only TC and its epimer were detected. In municipal wastewaters, only MIN was detected and the wastewater treatment resulted in a reduction of its level. The concentrations of MIN, before and after treatment, were 915.3 and 95.8 ng l^{-1}, respectively, indicating a reduction of 89.5%.

When the results of both seasons are compared, a seasonal influence was observed on the frequency of detection of TCs in hospital wastewaters and was evident especially for MIN and TC; however, these are only preliminary results. During spring, the levels found were higher, when compared to those found in samples collected during autumn. It should be noted that, during autumn, by the time when samples were collected, the weather was unusually warm and dry. It was also observed that MIN undergoes a more effective WWTP removal during autumn.

As far as compound stability in the environment is concerned, degradation of TC antibiotics is expected to be slow when exposure to sunlight is limited since they are photodegradable [21].

Moreover, degradation products are also of concern because they may have similar or higher toxicity than the parent compounds, for example, anhydrotetracycline (ATC) and epianhydrotetracycline causes Fanconi syndrome [22].

Only a few of the compounds were partially biodegraded under test conditions in aquatic systems; most of these compounds were persistent [21]. Moreover, previous studies indicate strong adsorption of TCs to clay material soil and sediments, throughout a wide range of environmental conditions given their complexation with cations [23]. For example, oxytetracycline (OTC), quinolone derivates, and sulfonamide antibiotics were found to be persistent in model marine aquaculture sediment [24]. TCs have been found in sediments, at concentrations that can reach 4.8 mg kg^{-1} [25].

Despite this fact, in the scientific literature, TCs have been reported in surface waters and wastewaters from WWTPs in different countries (Table 4), such as Sweden (0.064–2.480 μg l^{-1}) [26], Luxembourg (1.0–85.0 μg l^{-1}) [27], Canada (0.038–0.977 μg l^{-1}) [28], and USA where several studies demonstrate the presence of TCs [10, 29–33]. In particular, OTC was detected in Po and Lambro river (Italy) at concentrations up to 248.90 and 24.40 ng l^{-1}, respectively [34], and in an American WWTP influent (47 μg l^{-1}) and effluent (4.2 μg l^{-1}) [10] and surface waters (340 ng l^{-1})

Conclusions

A novel, simple, low-cost, and sensitive analytical strategy based on LC followed by postcolumn derivatization and fluorescence detection was developed for the routine analysis of TCs in environmental wastewaters. The accuracy and precision results testify to the efficiency of the proposed method for the intended purpose.

From the obtained results, the presence of MIN, DC, TC, and ETC in the analyzed samples is clear. The levels found ranged from 6 to 531.7 μg l^{-1} in hospital effluents, while its concentrations in WWTP ranged from 95.8 to 915.3 μg l^{-1}. These results show that hospital effluents have an important contribution for TC load in the WWTPs.

Moreover, data on the removal efficiency were achieved, ranging between 89.5% and 100%.

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5.9. Supplement V

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Determination of tetracycline antibiotics in hospital and municipal wastewaters in Coimbra, Portugal by liquid chromatography with fluorescence detection: occurrence and seasonal influence.

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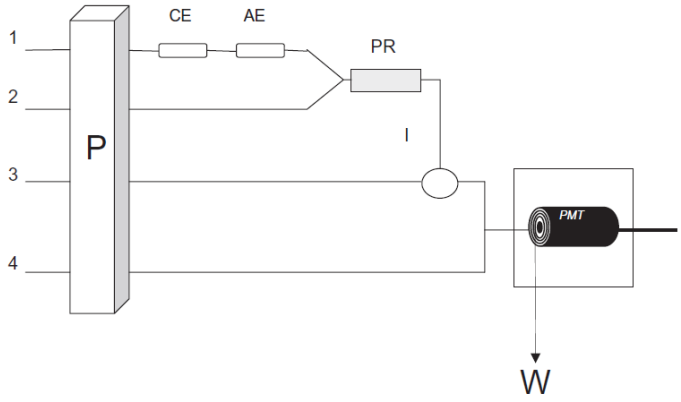

Figure 1. Manifold used for the determination of methomyl
1: Methomyl (2.25 mL min^{-1}); 2: Quinine 2×10^{-5} M/NaOH 0.2 M (2.25 mL min^{-1}); 3: Water (8.2 mL min^{-1}); 4: Cerium (IV) 2.5×10^{-4} M/H_2SO_4 0.13 M (3.7 mL min^{-1}); AE: anionic exchanger; CE: cationic exchanger; I: Injection Valve; P: Pump; PMT: Photomultiplier tube; PR: Photoreactor; W: Waste.

oxidation of the pesticide by Ce (IV) in sulphuric acid. Previous irradiation with UV light in basic medium was necessary. The manifold used for the determination and the optimized conditions are depicted in Figure 1.

As it is shown, quinine was mixed with the pesticide immediately before the photoreactor, due to its strong enhancing effect on CL, which was increased with this configuration. The effect of physical parameters such as flow rates, sample volume inserted and temperature was also studied. The results obtained showed that the most relevant parameters were the reaction and photodegradation times controlled by changing the corresponding flow rates.

The developed method was very fast (throughput of 123 h^{-1}) and allowed to carry out the determination in a range comprised between 2 and 80 μg L^{-1} of methomyl. The limit of detection was 0.05 μg L^{-1}, without need of enrichment steps, which was below of the regulatory limits (0.1 μg L^{-1}) established by the European Union [2]. The method was successfully applied to the determination of methomyl in two water samples (recovery rates of 98 ± 6 and 90 ± 5) previous removal of interferences with ionic exchangers.

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Determination of tetracycline antibiotics in hospital and municipal wastewaters in Coimbra, Portugal by liquid chromatography with fluorescence detection: occurrence and seasonal influence

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Introduction

The issue of human pharmaceutical products in the environment has recently raised a worldwide concern. It has been worldwide recognized that the environmental impact of pharmaceutical has to be evaluated, as requested by recent EU legislation (1).

In particular, the study of antibiotics residues is of special concern, mainly due to the associated problem of growing of antimicrobial resistant bacteria (2). In Portugal, antibiotics have been detected in hospital wastewaters, in wastewater treatment plants (WWTPs) (3–4), in river waters (5) and in piggeries environmental waters (6).

Tetracyclines (TCs) are a group of broad-spectrum antibacterial compounds active against a wide of human and animal pathogens and can be successfully determined by LC with UV detection that has low sensitivity, mass spectrometry that still requires costly instruments, and with fluorescence detection (FD), that is sensitive and selective (4).

WWTPs and hospital wastewaters are pointed out as the major source discharge of TCs into the environment demanding a survey to obtain a better knowledge on their occurrence and environmental fate.

The main goal of this work was the LC-FD determination of minocycline (MINO), tetracycline (TC), its epimer epitetracycline (ETC), and doxycycline (DC) residues in wastewaters from 4 hospitals and from WWTP, in Coimbra, Portugal, during spring,

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autumn and winter. Occurrence, seasonal influence and WWTP treatment efficiency were evaluated.

Experimental

TCs were separated on a C_8 column and derivatizated with magnesium acetate in pH 9.0 boric acid buffer, to produce a highly fluorescent complex. FD was performed at an excitation and emission wavelength of 360 and 500 nm, respectively (4).

Results

The LOQ was 15 µg/L for MINO, 0.5 µg/L for ETC and TC and 5 µg/L for DC. Mean recoveries ranged between 76% and 117%.

TC, MINO, ETC and DC were found in 42, 49%, 27% and 9% of samples, respectively. Hospital effluents were an important point source for MINO, for the other TCs a lower input was observed. The overall concentrations ranged from 6.0 to 531.7 µg/L. MINO was the only TCs detected in municipal wastewaters samples and its presence was observed in all of the 3 seasons. A high rate of removal, 89.5% to 100%, of the WWTP was observed. A seasonal influence was observed, since TCs levels found were higher during spring, when compared to those found in samples collected during winter, the lowest values were detected in autumn.

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Proteome analysis of diabetic-related proteins in rat kidney by FD-LC-MS/MS method

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Introduction

Diabetic nephropathy (DN) is a complication of diabetes mellitus which may lead to end-stage renal disease (ERSD), but the mechanism is not understood. Throughout its early course, DN has no symptoms. The first laboratory abnormality shows a positive sign by microalbuminuria test. However, at this stage, a kidney biopsy clearly shows DN. Because of the technological improvements in proteomics, there are a lot of applications in DN [1–2]. This study is designed to establish a new method to identify the differential expression of proteins in the kidney of control and streptozotocin (STZ)-induced diabetic rats in order to discover specific indicators for early diagnosis of DN.

Experimental

The 7-week-old male Spregue-Dawley rats were induced by intraperitoneal injection of sodium citrate buffer or STZ (80 mg/kg) (Sigma Chemical Co., St. Louis, MO, USA), and sacrificed after 1, 4, 12, and 24 weeks of administration. Urine microalbumin was determined by a commercial kit. The renal cortex was homogenized in 10 mM CHAPS and the contents of total protein were quantified by using the Bradford method [3]. A thiol-specific fluorogenic reagent, 4-[2-(dimethylamino)ethyl]-7-chloro-2,1,3-benzoxadiazole-4-sulfonamide (DAABD-Cl) (Tokyo Chemical Industry Inc., Tokyo, Japan) was utilized for the derivatization of proteins [4]. And then the derivatives were separated and collected by high performance lipid chromatograph (Hitachi, Tokyo, Japan) with fluorescence detection. The isolated derivatives will be further digested and identified by liquid chromatography tandem mass spectrometry (LC-MS/MS) (4000 Q TRAP, Applied Biosystems, Foster City, CA, USA) with the MASCOT database searching system. Figure 1 shows the experimental processes.

Results

Body weight and blood glucose were increased significantly after 4 days of STZ injection. After 1 week of administration, the expressions of approximately 7 proteins significantly changed. Only one pretein had significantly changed after 4 weeks of injection. However, after 12 weeks of STZ treatment, the number of altered proteins rose to 9. The results show that these proteins appeared earlier than microalbuminuria which changed significantly after 24 weeks of STZ administration and changed significantly between the control and diabetic rats.

Discussion

In the present study, we found that several proteins were altered under diabetic stages. In addition, the changes appeared before the increase of microalbumin in the urine. The identification of the altered proteins will be presented. The results may serve to find new biomarkers to improve early detection and new drug development in diabetic-related nephropathy.

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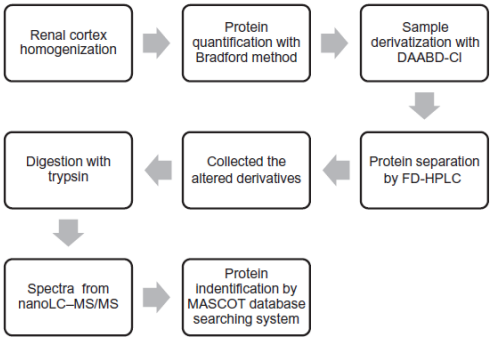

Figure 1. The experimental processes in the present study.

6. CONCLUSIONS

This doctoral thesis is based on the application of liquid chromatography for the analysis of antibiotics in environmental water samples. The focus of this doctoral thesis was on the group of fluoroquinolone and tetracycline antibiotics. An overview of recently published methods dealing with the determination of antibiotics in environmental samples is showing the increasing concern about the presence of antibiotics in the environment. Liquid chromatography with mass spectrometry detection was the widely used analytical technique for the determination of different groups of antibiotics in environmental samples. As the environmental samples are complex matrices and analytes occur there in very low concentration, SPE was the technique of choice for the concentration analytes and clean-up.

Further, new methods for the determination of fluoroquinolone and tetracycline antibiotics by liquid chromatography with fluorescence or mass spectrometry detection were developed. These methods were validated and applied for the analysis of wastewaters in order to monitor antibiotic residues in environmental waters during different season periods.

First study dealt with the determination of fluoroquinolone antibiotics in wastewater samples four hospitals and influent and effluent from wastewater treatment plant in Coimbra in Portugal. The samples were collected during spring and autumn season in order to evaluate the seasonal influence. Ciprofloxacin was found in all samples, followed by norfloxacin (79% samples) and ofloxacin (50% samples). Ciprofloxacin and ofloxacin were detected in very high concentrations in hospital wastewaters (up to 11 000 ng/L). This result is in agreement with the high usage of those antibiotics in human medicine. Norfloxacin was detected in hospital wastewaters in lower concentrations comparing to ciprofloxacin and ofloxacin. Enrofloxacin has not been detected in hospital wastewater as it is used only for veterinary purposes. Differences in antibiotic concentrations detected in wastewaters from different hospitals were observed. Moreover, a seasonal influence was evaluated as higher antibiotic concentrations were found in samples collected during spring. In addition, the samples from municipal wastewater treatment plant were analyzed. Norfloxacin, ciprofloxacin and enrofloxacin were detected and the efficiency of treatment process was evaluated. The reduction of antibiotics levels was observed in range 53% - 93% which is in agreement with other reported studies. The higher reduction efficiency was observed in early autumn season when the weather was unusually warm.

The second study dealt with the determination of fluoroquinolone antibiotics in samples from wastewater treatment plant of hospital in Hradec Králové. The samples

were collected during three seasons. Ciprofloxacin and ofloxacin were found in all samples, followed by norfloxacin (in 93% of samples). Ciprofloxacin and ofloxacin were detected in high concentration levels (up to 6600 ng/L). This result is in agreement with the Portuguese study confirming the usage those antibiotics in human medicine very frequently. Norfloxacin was detected in concentrations up to 1140 ng/L. Enrofloxacin has not been detected. A seasonal influence was clearly observed as the highest concentration levels were found in samples collected during late winter seasons. This might confirm the high usage antibiotics during this season. In addition, the efficiency of treatment process was evaluated. The reduction of antibiotics was observed in range 60 – 100%. There was no difference of the efficiency of treatment process in the dependence on season. Moreover, the samples from aeration tank were analyzed. The accumulation on antibiotics in aeration tank was observed confirming the suggestion that sorption of fluoroquinolones to sewage sludge is the main removal pathway for fluoroquinolones.

The third study dealt with the determination of tetracycline antibiotics in hospital wastewaters and samples from municipal wastewater treatment plant in Coimbra in Portugal. Minocycline and tetracycline were detected in 42% samples, followed by epi-tetracycline (25%) and doxycycline (8%) detection. Minocycline has been found in the highest concentration levels (up to 915 ng/L). The seasonal occurrence of tetracyclines was observed at higher concentration levels were found in samples during spring season. The samples from municipal wastewater treatment plant were analyzed. Only minocycline was detected and the reduction of 89.5% was observed.

The detection of antibiotic residues in the wastewaters shown the importance to continue in the monitoring of these compounds in the environment and evaluate the fate and possible effects on the aquatic organisms as well as human health. Moreover, the detection of antibiotics in effluent from wastewater required the improvement of the treatment process to avoid released of antibiotic residues in aquatic environment. Advance oxidation processes might be used as additional procedure during wastewater treatment to accomplish the treatment process.

A study of the degradation of fluoroquinolone antibiotic pefloxacin by advanced oxidation processes was done. The efficiency of ozonation, UV photolysis and their combination was compared. The results have shown the ozonation as the most efficient method for the degradation of pefloxacin. Although the total degradation of pefloxacin was observed by HPLC analysis, total organic carbon measurement shown no mineralization at all. This result might be explained by the transformation of pefloxacin into stable organic byproducts such as small organic acids. Moreover, the main degradation products of pefloxacin were identified. However, the toxicity data for these degradation products are missing and future research has to be done.

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